Universidade de Aveiro Departamento de Ciências Médicas
2020

José Carlos Martins Lopes

Endothelial progenitor cells and circulating endothelial cells in heart failure: a cross-sectional study

Células endoteliais progenitoras e células endoteliais circulantes na insuficiência cardíaca: um estudo transversal

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica do Doutor Fernando Manuel Tavares da Silva Ribeiro, Professor Adjunto da Escola Superior de Saúde da Universidade de Aveiro e co-orientação do Doutor Bruno Miguel Bernardes de Jesus, Professor Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro e Doutor Ramiro Daniel Carvalho de Almeida, Professor Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro.

This thesis was performed within a project financed by the FEDER under the new Partnership Agreement PT2020 within the project “POCI-01-0145-FEDER-030011” and by National Funds through FCT - Foundation for Science and Technology within the project "PTDC/MEC- CAR/30011/2017”.

Cofinanciado por:

COMPETE 2020 PORTUGAL 2020 UNIÃO EUROPEIA Fundo Europeu de Desenvolvimento Regional FCT Fundação para a Ciência e a Tecnologia

o júri

presidente

Profª. Doutora Ana Margarida Domingos Tavares de Sousa,
Professora Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro

Profº. Doutor Fernando Manuel Tavares da Silva Ribeiro,
Professor Adjunto da Escola Superior de Saúde da Universidade de Aveiro

Profª. Doutora Rita Maria Pinho Ferreira,
Professora Auxiliar do Departamento de Química da Universidade de Aveiro

agradecimentos

Após finalizar mais uma importante etapa da minha vida curricular, não podia deixar de agradecer a todas as pessoas que fizeram parte dela e que, de uma forma ou de outra, foram essenciais para este longo caminho.

Em primeiro lugar, gostaria de agradecer ao Professor Fernando Ribeiro pela orientação, motivação, tempo e dedicação prestada ao longo deste último ano.

Em segundo lugar, gostaria também de agradecer ao Professor Bruno Jesus e Professor Ramiro Almeida por toda a simpatia, generosidade e predisposição em ajudar.

À Professora Ana Cristina Gonçalves, pela realização da citometria de fluxo e por todas as críticas construtivas e disponibilidade.

A todos os meus colegas de laboratório, meus mentores, à Filipa, à Marisol, ao Manuel e à Magda pela disponibilidade, simpatia e por todos os conhecimentos laboratoriais que me transmitiram.

Aos meus colegas de Mestrado que ao longo deste ano e por todas as razões e mais algumas se tornaram grandes amigos, Adriana, Ricardo, Joana e Zé.

Aos amigos de Vila Real, Carolina, Joana e Tina por continuarem a amparar todas as minhas quedas e por festejarem todas as minhas pequenas conquistas.

À minha segunda família, Catarina, Daniela, Diogo, Jéssica, Nuno e Vieira um obrigado não chega por todo o apoio incondicional e amizade.

Por fim, dedico este trabalho aos meus pais e ao meu irmão, pelo apoio incondicional, incentivo, paciência e total ajuda na superação dos obstáculos que ao longo desta caminhada foram surgindo. Por todos os valores que me transmitiram e que são, em parte, responsáveis pela pessoa que sou hoje.

palavras-chave

Insuficiência cardíaca com fração de ejeção reduzida, células endoteliais circulantes, células endoteliais progenitoras, células estaminais hematopoiéticas, disfunção endotelial, dano vascular.

resumo

O presente trabalho teve como principal objetivo comparar os níveis de células endoteliais progenitoras (CEPs), células endoteliais circulantes (CECs) e células estaminais hematopoiéticas (CEHs) em circulação entre doentes com insuficiência cardíaca com fração de ejeção reduzida (ICFEr) e um grupo de adultos com fatores de risco cardiovasculares. Adicionalmente, os níveis das CEPs, CECs e CEHs foram comparados entre subgrupos em função da presença de fatores de risco (ex. diabetes) e da etiologia da insuficiência cardíaca. Inicialmente foram recolhidas amostras de sangue periférico de doentes com ICFEr (n = 42) e indivíduos da mesma faixa etária com fatores de risco cardiovasculares, mas sem qualquer doença cardiovascular estabelecida (n = 42). Em seguida, foi utilizada uma combinação de anticorpos nas amostras de sangue periférico para quantificação do número de CEPs, CECs e CEHs por citometria de fluxo. Doentes com ICFEr apresentaram níveis de CEPs ($5.28 \times 10^{-3} \pm 6.83 \times 10^{-4}$ % *vs* $7.76 \times 10^{-3} \pm 4.91 \times 10^{-4}$ %, $\boldsymbol{P} \leq 0.001$) e CECs ($5.11 \times 10^{-3} \pm 7.87 \times 10^{-4}$ % *vs* $6.51 \times 10^{-3} \pm 5.21 \times 10^{-4}$ %, $\boldsymbol{P} = 0.005$) significativamente inferiores aos indivíduos com fatores de risco cardiovasculares. Contudo, não foram encontradas diferenças significativas nos níveis de CEHs entre os dois grupos ($\boldsymbol{P} = 0.590$). Adicionalmente, observou-se que as CECs ($6.69 \times 10^{-3} \pm 6.38 \times 10^{-3}$ % *vs* $3.61 \times 10^{-3} \pm 2.71 \times 10^{-3}$ %, $\boldsymbol{P} = 0.057$) tendem a circular em maior número em doentes com ICFEr com etiologia isquémica comparativamente a doentes com ICFEr não isquémica. Doentes com ICFEr e com sobrepeso/obesidade apresentaram níveis de CEPs ($6.10 \times 10^{-3} \pm 4.78 \times 10^{-3}$ % *vs* $4.13 \times 10^{-3} \pm 3.55 \times 10^{-3}$ %, $\boldsymbol{P} = 0.043$) e CECs ($6.27 \times 10^{-3} \pm 5.66 \times 10^{-3}$ % *vs* $3.47 \times 10^{-3} \pm 3.54 \times 10^{-3}$ %, $\boldsymbol{P} = 0.019$) significativamente superiores comparativamente a doentes com ICFEr e com peso normal. Por último, dentro do grupo de indivíduos com fatores de risco cardiovasculares, indivíduos com dislipidemia apresentaram níveis de CECs ($7.74 \times 10^{-3} \pm 3.64 \times 10^{-3}$ % *vs* $5.34 \times 10^{-3} \pm 2.59 \times 10^{-3}$ %, $\boldsymbol{P} = 0.042$) significativamente superiores em comparação a indivíduos sem dislipidemia. Em conclusão, os principais resultados deste estudo indicam que o número de CECs e CEPs em circulação encontra-se significativamente reduzido em doentes com ICFEr comparativamente a indivíduos com fatores de risco para doenças cardiovasculares. As observações atuais em relação aos fatores de risco para doenças cardiovasculares sugerem que CEPs, CECs e CEHs desempenham um papel fundamental na sinalização e reparação do dano vascular e disfunção endotelial.

keywords

Heart failure with reduced ejection fraction, circulating endothelial cells, progenitor endothelial cells, hematopoietic stem cells, endothelial dysfunction, vascular damage.

abstract

The objective of the present thesis was to compare the levels of circulating endothelial progenitor cells (EPCs), circulating endothelial cells (CECs), and hematopoietic stem cells (HSCs) between patients with heart failure with reduced ejection fraction (HFrEF) and a group of subjects with cardiovascular risk factors. We also compared the levels of circulating EPCs, CECs, and HSCs between subgroups regarding the presence of cardiovascular risk factors (e.g. diabetes mellitus) and the etiology of heart failure (HF). To achieved this, whole peripheral blood was drawn from patients previously diagnosed with HFrEF (n = 42) and age-matched subjects presenting similar cardiovascular risk factors but without established cardiovascular disease (n = 42). Then, a combination of markers was used in peripheral blood samples in order to assess the number of circulating EPCs, CECs, and HSCs via flow cytometry analysis. Patients with HFrEF had significantly decreased levels of circulating EPCs ($5.28 \times 10^{-3} \pm 6.83 \times 10^{-4}$ % *vs* $7.76 \times 10^{-3} \pm 4.91 \times 10^{-4}$ %, $\boldsymbol{P} \leq 0.001$) and CECs ($5.11 \times 10^{-3} \pm 7.87 \times 10^{-4}$ % *vs* $6.51 \times 10^{-3} \pm 5.21 \times 10^{-4}$ %, $\boldsymbol{P} = 0.005$) compared to subjects with cardiovascular risk factors. However, levels of HSCs were not significantly different between the two groups ($\boldsymbol{P} = 0.590$). Additionally, CECs ($6.69 \times 10^{-3} \pm 6.38 \times 10^{-3}$ % *vs* $3.61 \times 10^{-3} \pm 2.71 \times 10^{-3}$ %, $\boldsymbol{P} = 0.057$) tended to circulate in higher number in patients with ischemic HF compared to patients with non-ischemic HF. Patients with HFrEF and diagnosed as overweight/obese had significantly higher levels of circulating EPCs ($6.10 \times 10^{-3} \pm 4.78 \times 10^{-3}$ % *vs* $4.13 \times 10^{-3} \pm 3.55 \times 10^{-3}$ %, $\boldsymbol{P} = 0.043$) and CECs ($6.27 \times 10^{-3} \pm 5.66 \times 10^{-3}$ % *vs* $3.47 \times 10^{-3} \pm 3.54 \times 10^{-3}$ %, $\boldsymbol{P} = 0.019$) when compared to patients with HFrEF presenting a normal weight. Lastly, when comparing subjects from the age-matched group, subjects with dyslipidemia had significantly higher levels of CECs ($7.74 \times 10^{-3} \pm 3.64 \times 10^{-3}$ % *vs* $5.34 \times 10^{-3} \pm 2.59 \times 10^{-3}$ %, $\boldsymbol{P} = 0.042$) compared to subjects without dyslipidemia. In conclusion, the main result of this study is that the circulating levels of EPCs and CECs were significantly decreased in patients with HFrEF in comparison to subjects with cardiovascular risk factors. The current observations regarding cardiovascular risk factors suggest that EPCs, CECs, and HSCs play an important role in the detection and repair of vascular damage and endothelial dysfunction.

LIST OF CONTENTS

Chapter IV: Results

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACE	→	angiotensin-converting enzyme
ACE-I	→	angiotensin-converting-enzyme inhibitors
ADH	→	antidiuretic hormone
AHA	→	American Heart Association
ANP	→	atrial natriuretic peptide
ARB	→	angiotensin II receptor blocker
ARNI	→	angiotensin-receptor neprilysin inhibitor
BMI	→	body mass index
BNP	→	brain natriuretic peptide
CAD	→	coronary artery disease
CEC	→	circulating endothelial cell
cGMP	→	cyclic guanosine monophosphate
CHF	→	congestive heart failure
CO	→	cardiac output
CRT	→	cardiac resynchronization therapy
CST	→	Chester step test
DMAP	→	damage-associated molecular patterns
ECG	→	echocardiography
EDTA	→	ethylenediaminetetraacetic acid
EDHF	→	endothelium-derived hyperpolarizing factor
eNOS	→	nitric oxide synthase
ET-1	→	endothelin-1
EPC	→	endothelial progenitor cell
ESC	→	European Society of Cardiology
FSC	→	forward scatter
FSC-A	→	forward scatter-area
FSC-H	→	forward scatter-height
GCSF	→	cytokines of granulocyte colony-stimulating factor
HbA1c	→	hemoglobin A1c
HDL	→	high-density lipoproteins
HF	→	heart failure
HFmrEF	→	heart failure with mid-range ejection fraction
HFpEF	→	heart failure with preserved ejection fraction
HFrEF	→	heart failure with reduced ejection fraction
HR	→	heart rate
HSC	→	hematopoietic stem cell

HSPC	→	hematopoietic stem progenitor cell
ICD	→	implantable cardioverter-defibrillator
ISHAGE	→	International Society of Hematotherapy and Graft Engineering
JVP	→	jugular venous pressure
LDL	→	low-density lipoproteins
LV	→	left ventricle
LVEF	→	left ventricular ejection fraction
LVSD	→	left ventricular systolic dysfunction
MI	→	myocardial infarction
MMP-9	→	matrix metalloproteinases-9
MRA	→	mineralocorticoid receptor antagonists
MRI	→	magnetic resonance imaging
MUGA	→	multiple-gated acquisition scanning
NADPH	→	nicotinamide adenine dinucleotide phosphate
NO	→	nitric oxide
$ONOO^-$	→	peroxynitrite
NP	→	natriuretic peptides
NT-proBNP	→	N-terminal fragment of brain natriuretic peptide
NYHA	→	New York Heart Association
PGH_2	→	prostaglandin H2
PGI_2	→	prostacyclin
PKG	→	cGMP-dependent protein kinase
RASS	→	renin-angiotensin-aldosterone system
ROS	→	reactive oxygen species
RV	→	right ventricle
SDF-1	→	stromal cell-derived factor 1
sGC	→	soluble guanylyl cyclase
SNS	→	sympathetic nervous system
SSC	→	side scatter
STEMI	→	ST-segment-elevation myocardial infarction
T2DM	→	type II diabetes mellitus
VAD	→	ventricular assist device
VEGF	→	vascular endothelial growth factor
VO_2 peak	→	peak of oxygen uptake
vWf	→	von Willebrand factor

CHAPTER I: BACKGROUND

1.1 Heart Failure

1.1.1 Heart Failure in numbers

According to the World Health Organization (WHO), cardiovascular diseases represent the leading cause of global mortality, taking an estimated 17.9 million lives each year, representing 31% of all deaths worldwide[1]. One in nine deaths includes heart failure (HF) as a contributing cause, affecting 26 million people across the globe, with more than half of these patients dying within 5 years of admission[2,3]. Over the past few years, the survival rate in patients with HF has improved in many parts of the world, nevertheless, in low-and middle-income countries, the one-year death rate remains high, reaching 34% in Africa, 23% in India, 15% in South East Asia, 9% in South America, and 7% in China[4,5]. Due to its high and increasing prevalence rate, HF constitutes an enormous economic burden for the healthcare system in developed countries, estimated at 108 billion dollars annually, representing about 2% of the total budget for health[6,7]. In 2014, the average annual cost per patient with HF in Portugal was estimated to be 1159 euros, making the overall expenses for the Portuguese health care system reach 289.4 million euros[8]. The prevalence of HF in the adult Portuguese population was estimated at 4.36%, reaching 12.67% in the 70-79-year age group and 16.14% over 80 years old[9]. A study performed by the Faculty of Medicine, University of Lisbon, anticipates that the deaths caused by HF in Portugal are estimated to increase by 73% in the next 20 years, which equivalates to 8112 deaths by 2036[10].

In recent years, the prevalence of cardiovascular risk factors among the Portuguese population has been rapidly increasing[11]. According to a report published by the Dr. Ricardo Jorge National Health Institute, 68% of the Portuguese population has at least two cardiovascular risk factors, such as hypertension (43.1%), diabetes mellitus (8.9%), low levels of physical activity (29.2%), alcohol abuse (18.8%), and smoking habits (25.4%)[12]. Furthermore, Portugal is one of the European countries that has registered a faster increase in the prevalence of overweight/obesity, affecting 53% of the population in the 18-64-year age group[11].

1.1.2 Definition and etiology of Heart Failure

HF is a complex clinical syndrome defined as the inability of the heart to supply the required amount of blood and oxygen to meet the body's metabolic needs and accommodate the systemic venous return, at rest or during exercise[13–15]. The ventricles, which are the main pumping chambers of the heart, become weaken and/or stiff leading to abnormalities in the systolic and/or diastolic function, culminating in a reduction of cardiac output (CO) and/or elevation of intracardiac pressures[16,17]. The CO is defined as the volume of blood pumped by the heart per unit of time, or in other words, "the amount of work performed by the heart in response to the body's need for oxygen"[18]. Pathophysiologically, patients with HF often display a normal CO until later stages of the disease, when the product of stroke volume and heart rate (HR) becomes insufficient to support the execution of simple activities of daily living nor can it increase sufficiently to meet the high metabolic demands from moderate exercise[19].

According to the American Heart Association (AHA), the development of HF often originates as the result of an underlying myocardial disease, leading to downstream deleterious effects at the myocardial, neurohormonal, and endothelial levels (**Figure 1**)[20,21]. The impairment of cardiac function can arise through a variety of cardiac disorders, such as myocardial infarction (MI), coronary artery disease (CAD), cardiomyopathy (including dilated, hypertrophic, and restrictive), myocarditis, endocardial or pericardial disorders, and congenital heart defects[22,23]. However, valvular disorders, severe lung disease (e.g. emphysema), and abnormalities in the heart rate/rhythm can also result in cardiac malfunction[15]. The current definition of HF restricts itself to stages at which clinical symptoms are apparent, excluding asymptomatic patients that may have structural or functional cardiac abnormalities, which are established precursors of HF[24]. Nevertheless, the current HF guidelines emphasize the importance of these precursors, as they signalize the earlier stages in the progression to clinically evident HF and initiation of the treatment at the precursor stage, which is a valuable tool to reduce the mortality rate in patients with asymptomatic left ventricular systolic dysfunction (LVSD)[25].

HF is a clinical syndrome characterized by the manifestation of a spectrum of symptoms and signs, such as dyspnea, fatigue, elevated jugular venous pressure (JVP), pulmonary congestion, peripheral edema, tachycardia, and limitations in exercise

tolerance[26,27]. According to the severity of the symptoms, HF patients can be placed into one of four categories based on their limitations during physical exercise. The most commonly used classification system is the New York Heart Association (NYHA) Functional Classification. For instance, a patient with minimal or no symptoms but a severe obstruction of the left main coronary artery can be classified with a function capacity I) and objective assessment D)[28].

HF is the final common pathway of numerous cardiovascular risk factors and most forms of heart disease[29]. There are several risk factors associated with HF, some of them cannot be controlled and/or changed, such as family history, age, gender, and ethnicity[30]. However, others are modifiable, meaning that actions can be taken to changed them, such as high blood pressure, unhealthy blood cholesterol levels, diabetes mellitus, obesity, tobacco use, physical inactivity, drug abuse, and excessive alcohol consumption[31,32].

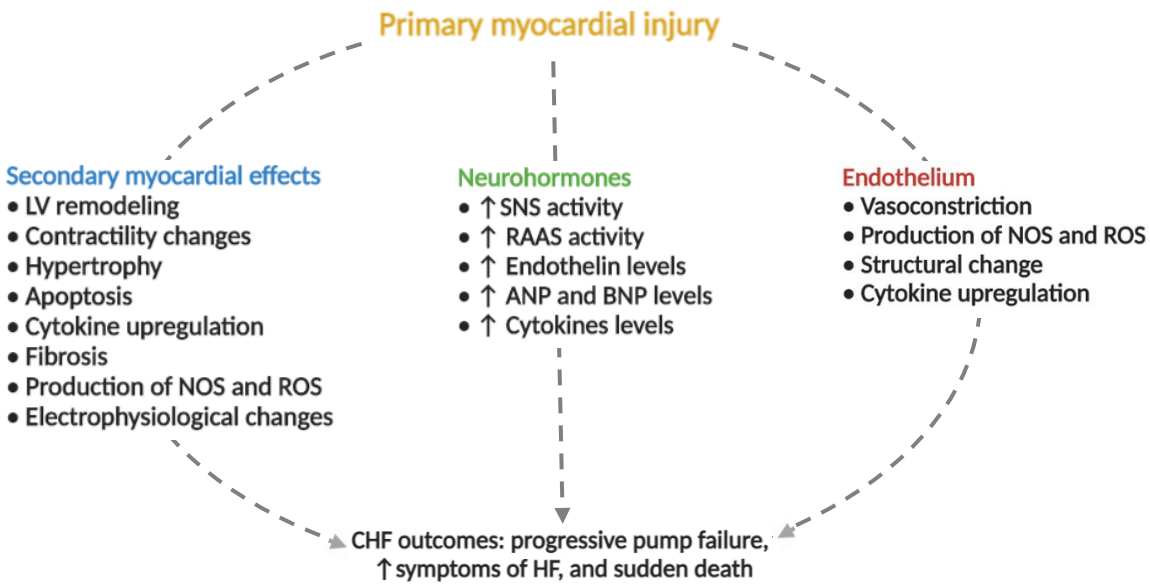

Figure 1. Pathophysiology of HF. Initial myocardial injury leads to downstream effects at the myocardial, neurohormonal, and endothelial levels; resulting in a decline of the ventricle function, worsening of HF symptoms, and ultimately sudden cardiac death. Myocardial injury causes a compensatory upregulation of the SNS and the RAAS. Initially, these responses can be helpful to maintain CO and myocardial contractility, however, the chronic activation of these systems becomes part of the disease process itself, leading to LV remodeling, myocardial fibrosis, and apoptosis. Additionally, pathological endothelin and cytokine upregulation can also ensue, leading to further complications, such as vasoconstriction and production of NOS and ROS. **ANP**, atrial natriuretic peptide; **BNP**, brain natriuretic peptide; **CHF**, congestive heart failure; **HF**, heart failure; **LV**, left ventricle; **NOS**, nitric oxide synthase; **RAAS**, renin-angiotensin-aldosterone system;

ROS, reactive oxygen species; **SNS**, sympathetic nervous system. The figure was produced using Biorender. Adapted from Bloom *et al.*[33].

1.1.3 The terminology of Heart Failure

1.1.3.1 Left/right-sided or left/right ventricular Heart Failure

The left ventricle (LV) is responsible for supporting most of the heart's pumping power and, for that reason, plays a key role in the preservation of the normal function of the heart[34]. Left-sided or left ventricular HF occurs when the heart no longer pumps enough blood through the body and, as a result, blood "backs up" in the pulmonary veins[35]. Chronic or poorly controlled hypertension, CAD, and arrhythmias are part of a broad spectrum of pathologies implied in the development of left-sided HF by increasing cardiac workload, compromising the ventricular contractility, and decreasing the CO[36].

The right ventricle (RV) is responsible for pumping the "used" blood that returns to the heart back into the lungs to be replenished with oxygen[37]. Right-sided or right ventricle HF occurs when the right side of the heart is too weak to pump blood efficiently to the lungs. It is also known as congestive heart failure (CHF) because blood slows as it flows out of the heart, returning through the veins. This backlog of blood causes congestion in the body's tissues, often resulting in a build-up of fluid[35].

1.1.3.2 Heart Failure with preserved, mid-range, and reduced ejection fraction

The main terminology used to describe HF is based on the percentage of blood pumped from the LV in each contraction (systole), defined as left ventricular ejection fraction (LVEF) [33,38]. Historically, patients with HF were divided into two clinically distinct syndromes: HF with reduced ejection fraction (HFrEF) and HF with preserved ejection fraction (HFpEF)[39]. HFrEF occurs as the LV losses the ability to normally contract, not generating enough force to circulate blood through the body[40]. On the other hand, in HFpEF the LV loses its ability to relax normally[41]. As a result, the heart cannot properly fill with blood during the resting period between each beat (diastole)[35]. In general clinical practice, normal LVEF is typically considered as ≥ 50% (HFpEF) and HFrEF with LVEF < 40%[40]. More recently, the 2016 European Society of Cardiology (ESC) Guidelines for the diagnosis

and treatment of acute and chronic HF acknowledged the existence of a "grey area" between HFrEF and HFpEF, in which patients with LVEF in the range 40-49% were included, hence the term HF with mid-range ejection fraction (HFmrEF)[41].

Over the years, the majority of studies have focused their attention understanding the burden of comorbidities among patients with HFrEF *versus* HFpEF, leaving the impact of comorbidities among patients with HFmrEF poorly understood[42]. The occurrence of HFrEF is generally preceded by acute or chronic loss of cardiomyocytes due to ischemia, genetic cardiomyopathies, myocarditis, or valvular disease. However, HFpEF is more closely associated with chronic inflammation and a higher prevalence of noncardiac comorbidities, which represents important cardiovascular risk factors (**Figure 2**)[43].

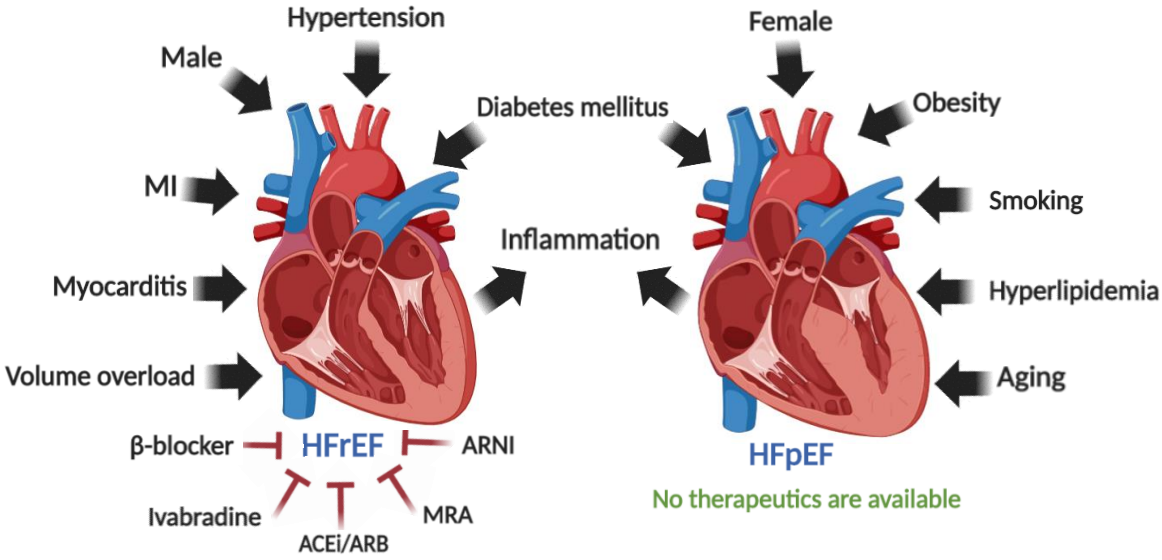

Figure 2. Risk factors and comorbidities involved in the development of each type of HF. HFrEF is more common in men and is preceded by volume overload, myocarditis, MI, and diabetes mellitus. However, HFpEF is more frequent in women and is often preceded by chronic comorbidities, such as obesity, hypertension, hyperlipidemia, diabetes mellitus, and aging. In patients with HFrEF, specific medication, such as β-blockers, ACEi/ARB agents, ARNI, ivabradine, and MRAs have been proven to reduce mortality. On the other hand and to be the best of our knowledge, there are currently no effective validated therapies for the reduction of morbidity and mortality in patients with HFpEF. **ACEi**, angiotensin-converting enzyme inhibitor; **ARB**, angiotensin II type 1 receptor blocker; **ARNI**, angiotensin receptor neprilysin inhibitor; **HFrEF**, heart failure with reduced ejection fraction; **HFpEF**, heart failure with preserved ejection fraction; **MI**, myocardial infarction; **MRA**, mineralocorticoid receptor antagonist. The figure was produced using Biorender. Adapted from Zakeri *et al.*[44].

1.1.4 Compensatory mechanisms

Several natural compensatory mechanisms are "called into action" in failing hearts in order to ensure adequate blood pressure and volume to maintain the functional capacity of the heart preserved or minimally depressed[45]. The main neurohormonal mechanisms activated in response to reduced systemic perfusion are the sympathetic nervous system (SNS), the renin-angiotensin-aldosterone system (RAAS), and the antidiuretic hormone (ADH)[46]. Activation of the sympathetic system in HF occurs via low- and high-pressure baroreceptors, which maintain the CO levels by increasing HR, myocardial contractility, and peripheral vasoconstriction[47]. Sustained sympathetic stimulation activates the RAAS and other neurohormones, leading to an increase in the concentrations of angiotensin II, renin, and aldosterone[48]. Angiotensin II is a potent vasoconstrictor of the renal and systemic circulation, responsible for promoting the release of aldosterone, leading to an increase in the retention of sodium and water by the kidneys, as well as a rise in the excretion of potassium[49]. The retention of salt and water increases the volume of blood in the bloodstream and helps to maintain blood pressure[50]. To offset the excessive activation of RAAS, which may lead to constriction of the peripheral blood vessels, the activation of vasodilatory molecules is increased, such as nitric oxide (NO), prostaglandins (PGE_2 and PGI_2), and atrial/brain natriuretic peptides (ANP, BNP)[51]. However, the excessive sympathetic activity can lead to cardiac myocyte apoptosis, hypertrophy, and focal myocardial necrosis[52].

The natriuretic peptides (NPs) are a group of peptide hormones with a vital role in the physiological control of cardiovascular functions[53]. The release of these peptides by the heart occurs in response to atrial and ventricular distension, as well as by neurohumoral stimuli, generally increased in response to HF[54]. The main physiological role of NPs is to oppose the effects of angiotensin II on systemic vascular resistance, sympathetic tone, aldosterone activity, and cardiac remodeling. Activation of this system aims to act as a counter-regulatory system for the RAAS (**Figure 3**)[55].

Compensatory mechanisms help the body adjust to the outcomes of HF in the earlier stages, making it more difficult for an early diagnose since the patient does not reveal any symptoms[56]. However, chronic stimulation of these mechanisms can have detrimental effects on the heart, leading to progressive cardiac dilation, alteration in cardiac structure,

and development and subsequent progression into chronic HF. It can also affect the kidneys and peripheral vasculature[57].

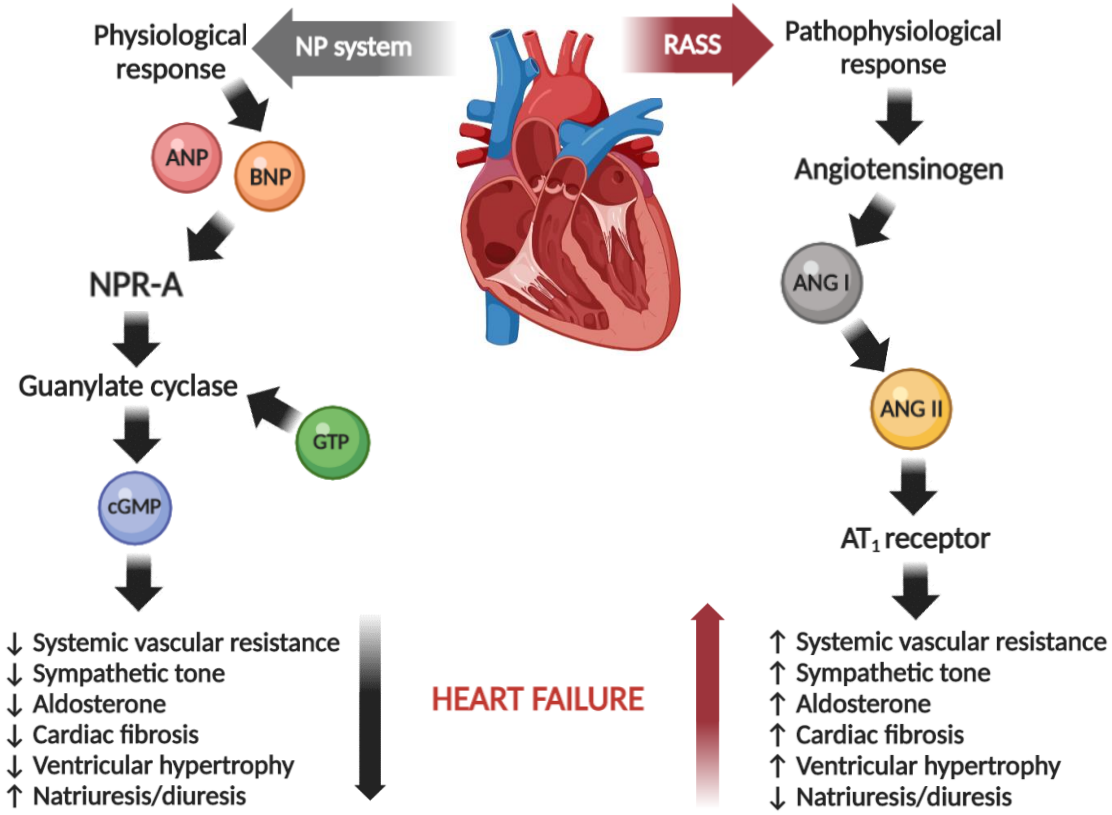

Figure 3. Counter-regulatory neurohormonal systems in HF. The NP system is comprised of three homologous peptides: ANP, BNP, and CNP, which can bind to two different biologically active receptors (NPR-A and NPR-B). Both ANP and BNP bind to the NPR-A and CPN binds to the NPR-B. NPR-A coupled with guanylate cyclase, which catalyzes the synthesis of cGMP, results in a decrease in systemic vascular resistance and central venous pressure, and an increase in natriuresis. In the RASS, the precursor polypeptide angiotensinogen is produced in the liver. Renin catalyzes the synthesis of Ang I by angiotensinogen, and Ang I is further converted into Ang II. At the kidney, Ang II binds to AT_1, leading to an increase in systemic vascular resistance and central venous pressure, a decrease in natriuresis, as well as a stimulus of the synthesis of aldosterone. **ANG I**, angiotensin I; **ANG II**, angiotensin II; **ANP**, atrial natriuretic peptide; **AT_1**, angiotensin type 1; **BNP**, brain natriuretic peptide; **cGMP**, cyclic guanosine monophosphate; **CNP**, C-type natriuretic peptide; **GTP**, guanosine-5'-triphosphate; **NP**, natriuretic peptide; **NPR-A**, natriuretic receptor-A; **NPR-B**, natriuretic receptor-B; **RAAS**, renin-angiotensin-aldosterone system. The figure was produced using Biorender. Adapted from Langenickel *et al.*[55].

1.1.5 Diagnostic

Timely diagnosis has become increasingly important as new drug treatment opportunities for HF have emerged with the potential to decrease symptoms, delay disease progression, enhance survival rate, and improve the quality of life[58]. However, in the early stages of HF, symptoms are often non-specific, making it difficult to discriminate between HF and other conditions[59]. In addition, patients often display several coexisting conditions, treated with several different medications, which can complicate the evaluation of the patient clinical status[60]. According to the ESC guidelines, the procedure to diagnose HF in a non-acute setting is presented as follows: first is estimated the probability of HF based on the patient's prior clinical history, the presenting symptoms, physical examination, and resting echocardiography (ECG). If all elements are normal, HF is most unlikely. If at least one element is abnormal, plasma NPs should be measured[38]. A proper detailed clinical history should not only include a careful assessment of the symptoms but also attempt to identify the etiology and common triggering factors of HF[61]. The assessment of clinical symptoms and signs of HF can be attained through a physical examination, being the major physical findings: bilateral ankle edema, laterally displaced left ventricular impulse, systolic murmur, jugular venous dilation, and crackles during inhalation[62]. The assessment of HF can also be achieved through lab work, which may include complete blood count, urinalysis test, liver function test, and complete metabolic profile for levels of serum electrolytes (including calcium and magnesium), blood urea nitrogen, serum creatinine, glucose, fasting lipid profile, and thyroid-stimulating hormone[63].

The NPs are the most widely studied and used biomarkers in HF[64]. BNP is a cardiac-derived hormone produced in the ventricular myocardium and released into the circulatory system in response to increased cardiac wall stress[65]. ProBNP, a 108-amino acid polypeptide, is segregated into the ventricles in response to volume expansion and pressure overload[66]. After his release, proBNP breaks down into two cleaved forms, the 76-peptide, biologically-inert N-terminal fragment, NT-proBNP, and the 32-peptide, biologically-active hormone, BNP[67]. The plasma concentration of BNP and its N-terminal fragment can be used as an initial diagnostic and prognostic biomarker in the management of HF, holding a 70/99% sensitivity and a specificity of 99/85%, respectively[68]. However, noncardiac conditions can

be responsible for elevated BNP in the bloodstream, such as kidney failure, acute large pulmonary embolism, high blood pressure, and chronic hypoxia[65].

BNP and NT-proBNP are described as the benchmarks against which other biomarkers are compared[69]. HF biomarkers can be organized into different categories: myocardial stress/injury, neurohormonal activation, remodeling, and comorbidities[70]. For instance, cardiac troponins are often elevated in patients with HF, as a result of myocardial injury[71]. However, they are not useful for the diagnosis of HF as they are not specific for these patients and are increased in other conditions that cause increased stress on the heart muscle[72]. Similarly, cardiac biomarkers, such as galectin-3, soluble suppression of tumourigenicity-2, and pro-adrenomedullin are also increased in patients with HF, as well as in other conditions[73]. Other diagnostic procedures for HF include chest X-rays, exercise stress test, magnetic resonance imaging (MRI), cardiac catheterization, and multiple-gated acquisition scanning (MUGA)[74].

1.1.6 Treatments

The current standard therapeutic approach for HF firmly focuses on improving patient's clinical status, functional capacity, and quality of life; reducing the frequency of hospitalizations, as well as decreasing associated mortality[75]. However, despite these efforts, the HF one-year mortality rate has only slightly declined and the five-year mortality rate has not declined in the last 10 years[76]. The treatment of HF can be divided into two categories: healthy lifestyle changes and pharmacological therapy. Promoting healthy lifestyle changes is crucial for reducing the burden on the heart muscle. Some of these changes may include restriction of sodium and fluid intake, monitoring of blood pressure, tracking symptoms, managing stress, losing or maintaining weight, cessation of smoking or alcohol, avoiding or limiting caffeine consumption, eating a heart-healthy diet, and being physically active[77]. Pharmacological therapy for HF patients is based on the administration of β-blockers, angiotensin-converting enzyme (ACE) inhibitors (ACEi)/angiotensin II receptor blockers, angiotensin-receptor neprilysin inhibitors (ARNIs), I_f channel blocker or inhibitor, aldosterone antagonists, and diuretics[78].

Some of the effects of the administration of this medication include reduction of blood pressure, slow HR, a decrease of symptoms, improvement of blood flow and heart function, reduction of workload on the heart, a decline of HF hospitalization, and reduction of mortality[79]. However, some of these medications have potential side effects, such as bradycardias, severe hypotension or renal insufficiency, and reduction of forced expiratory volume[80]. In addition to pharmacological therapy, novel surgical techniques to alleviate the underlying problem that led to HF have been introduced, such as coronary revascularization, ventricular restoration, heart valve repair for ischemic mitral incompetence, left ventricular aneurysmectomy, mechanical circulatory assist devices (ventricular assist devices (VADs)), implantable cardioverter-defibrillators (ICDs), cardiac resynchronization therapy (CRT) or biventricular pacing[81]. In more severe cases of HF, the main alternatives are heart transplantation or permanent mechanical circulatory support[82].

Physical activity is acknowledged as a fundamental adjutant therapy in cases of chronic HF[83]. In the scientific community, data related to the protective benefits of exercise in HF patients have become a hot topic, with the number of reports on PubMed rapidly increasing in the last few years[84]. Countless studies have shown numerous benefits of regular physical activity in patients with HF, such as a decrease in morbidity and mortality, an increase in life expectancy, reduction of symptoms, and risk of developing cardiovascular and respiratory diseases[85]. Physically active individuals demonstrate lower systolic and diastolic blood pressure, more favorable plasma lipoprotein profile, higher insulin sensitivity, an increase of myocardial oxygen supply, improved myocardial contraction and electrical stability, an increase of coronary collateral circulation and myocardial capillary density, favorable hemostatic mechanisms, lower concentration of inflammatory markers, stabilization of atheromatous plaques, improvements in endothelial function, an increase of vagal tone, and a decrease of sympathetic nervous system activity[86–88]. According to the AHA, 30 minutes of walking for at least 5 days/week at moderate-intensity, weekly 75 minutes of vigorous-intensity aerobic physical activity, or a combination of both, are the general physical activity recommended for optimal cardiovascular health[89,90].

1.2 The vascular endothelium

1.2.1 The role of the vascular endothelium

The vascular endothelium, once considered to be a simple passive barrier, is now recognized as a complex, highly selective, and metabolically active tissue[91,92]. It is comprised of a monolayer of endothelial cells lining the lumen of all blood vessels, separating the vascular wall from the circulation and the blood components[93].

The healthy endothelium responds to several humoral, neural, and hemodynamic stimuli; becoming a major player in the modulation of plasma permeability, vascular signaling and angiogenesis, blood flow regulation, and inflammatory response[94]. Indeed, the endothelium has a tightly regulated balance between pro- and antioxidants, pro- and anti-inflammatory molecules, pro- and antiproliferative factor, and pro- and antithrombotic signals[95]. In addition, endothelial cells play a key role in the maintenance of vascular homeostasis by synthesizing and releasing vasoactive substances, and by doing so, the endothelium has a profound effect on the overall function of the cardiovascular system[96,97]. The vasodilation is mainly mediated by factors, such as NO, endothelium-derived hyperpolarizing factor (EDHF), and prostacyclin (PGI_2), while a vasoconstrictor state is mediated by factors, such as endothelin-1 (ET-1), angiotensin II, thromboxane A_2, and prostaglandin H2 (PGH_2)[98]. Among these endothelial-derived factors, NO is considered to be the most important and the most well-characterized vasodilator molecule produced by the endothelium[99]. NO has a spectrum of biological properties that play a pivotal role in preserving the homeostasis of the vascular wall by inhibiting platelet adhesion and aggregation, leukocyte adhesion, inflammation, vascular smooth muscle cell migration and proliferation, and oxidative stress[100]. Essentially, NO is synthesized and released from the amino acid L-arginine through the activity of the calmodulin-dependent enzyme NO synthase (eNOS), which is continuously produced and released by endothelial cells under the influence of chemical agonists acting on specific endothelial chemoreceptors or by mechanical forces, such as shear stress, ischemia, and changes in temperature (**Figure 4**)[98,101]. Hemodynamic shear stress, generated by blood flow, triggers vasodilation mediated for the most part by increased endothelial eNOS activity, leading to a rapid rise in NO production[102]. Chemical stimuli include acetylcholine, bradykinin, thrombin, and serotonin[103]. Under normal, basal conditions in blood vessels, NO diffuses into the smooth

muscle cell promoting the activation of soluble guanylyl cyclase (sGC) and the production of cyclic guanosine monophosphate (cGMP). The increase in cGMP activates cGMP-dependent protein kinases (PKGs), which leads to smooth muscle cell relaxation via several mechanisms, such as alteration of membrane potential and intracellular calcium levels, activation of myosin light chain phosphatases, and regulation of smooth muscle cell contraction[104]. NO also has antiplatelet effects and can downregulate inflammatory pathways and the generation of ET-1, a powerful vasoconstrictor, which also holds pro-inflammatory, pro-oxidant, and pro-proliferative properties[105].

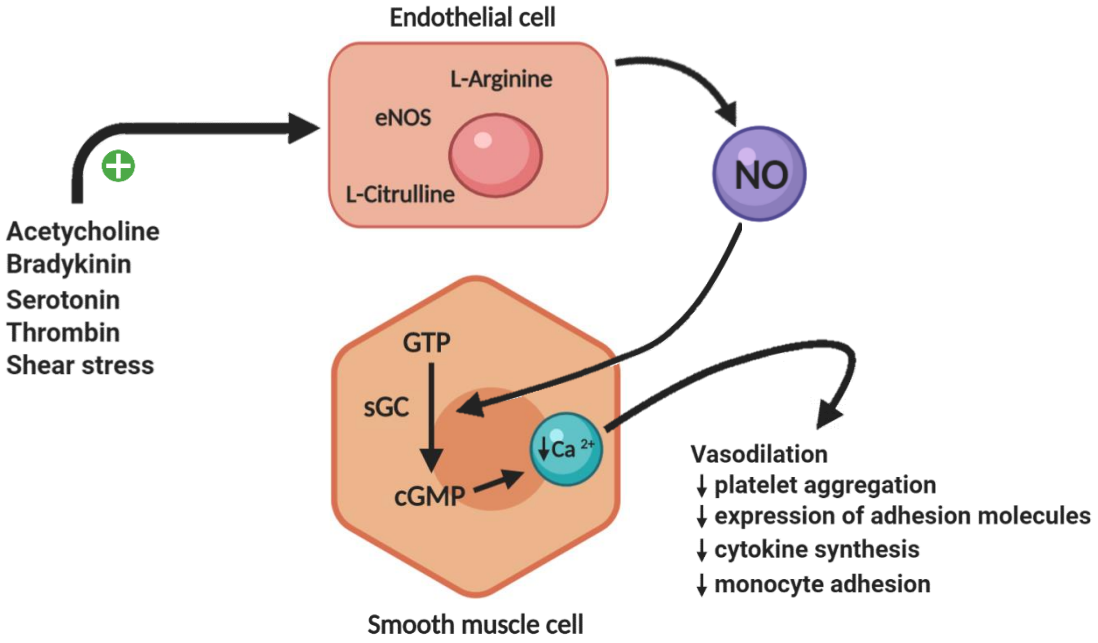

Figure 4. Synthesis of NO. The production of NO is stimulated by several stimulus. In endothelial cells, eNOS catalyzes the oxidation of L-Arginine to NO and L-Citrulline. NO diffuses into vascular smooth muscle cells promoting vasodilation by activating sGC, thereby increasing intracellular cGMP. **cGMP**, cyclic guanosine monophosphate; **eNOS**, endothelial nitric oxide synthase; **GTP**, guanosine triphosphate; **NO**, nitric oxide; **sGC**, soluble guanylate cyclase. The figure was produced using Biorender. Adapted from Ferreira *et al.*[106].

1.2.2 Endothelial cells

1.2.2.1 Endothelial progenitor cells

More than two decades ago, Asahara and colleagues published a landmark paper identifying a hematopoietic population of cells isolated from adult peripheral blood and capable of differencing *in vitro* into endothelial progenitor cells (EPCs)[107,108]. Furthermore, the same study observed that EPCs contributed to neovascularization and ischemic rescue when incorporated into an animal model of peripheral limb ischemia[109]. Since their discovery, the precise cellular definition and characterization of EPC remains unclear and the subject of considerable debate[110]. Human EPCs have been defined as bone marrow-derived cells, expressing a variety of cell surface markers similar to those expressed by vascular endothelial cells and with the capacity to adhere to the endothelium at sites of hypoxia/ischemia, contributing to the formation of new blood vessels[111,112].

In the last decade, circulating EPCs have taken center stage in the expanding field of vascular biology, as more and more studies have highlighted the importance of EPCs as a major contributor to vasculogenesis, angiogenesis, preservation of the normal vascular homeostasis, and repair (**Table 1**)[113–115]. In healthy individuals, EPCs have been shown to represent between 0.01% and 0.0001% of the total fraction of monocytes in circulation, with the majority of these cells located in the bone marrow[116]. However, under the stimulus of physiological or pathological factors, bone marrow-derived EPCs can be mobilized into the bloodstream where they participate in the repair of damaged tissue and ischemia by angiogenesis[117]. The process of EPCs mobilization and migration from the bone marrow is regulated by a variety of growth factors, enzymes, ligands, and surface receptors[118].

Endothelial damage, following ischemia-reperfusion injury or heart infarction, promotes the mobilization of several mediators, such as cytokines of granulocyte colony-stimulating factor (GCSF), matrix metalloproteinases-9 (MMP-9), vascular endothelial growth factor (VEGF), stromal cell-derived factor 1 (SDF-1), and eNOS[119]. The increased production of EPC-mobilizing factors, in particular, activated MMP-9, leads to EPCs release and mobilization from the bone marrow into the peripheral circulation[120]. Circulating EPCs can migrate towards injured endothelium or inflamed tissue, proliferate, and differentiate into mature endothelial cells, thus improving blood flow and regulating vascular repair via paracrine mechanisms[121].

1.2.2.2 Circulating endothelial cells

Circulating endothelial cells (CECs) are mature endothelial cells that have been shed from the lining of the vessel walls into the bloodstream in response to endothelial damage[122,123]. Although CECs were first described over 30 years ago by Bouvier and Hladovec, only recently have received in-depth attention from clinical researchers as an additional marker for the assessment of vascular integrity[124,125]. A few years later, an increased number of CECs was observed in different models of endothelial damage, both in animals, such as shock by E. Coli endotoxin and in humans with cardiovascular risk factors (e.g. hypertension) after acute MI, and with immunosuppression[126]. However, contradictory results were reported by these groups, due to the variety of cell fractions studied, differences in cell identification, and distinct methods for measurement of cell concentration, based on physical properties, such as size or density[124].

Endothelial cells provide the physical interface between the bloodstream and the surrounding tissue, regulating nutrient and blood component traffic, and participating in many physiologic events such as hemostasis, inflammation, and angiogenesis[127]. Under normal physiological conditions, these cells would be expected to remain in the endothelium, with perhaps a very low level of cell loss into the blood, with consequent clearance by the reticuloendothelial system[128]. The increased number of CECs, disconnected from the vessel wall in response to tissue ischemia, is thought to be due to a variety of factors such as oxidative stress, infectious agents, cytokines, proteases, anti-endothelial cell antibodies, disturbed flow-induced p53, and ERK5 SUMOylation[129]. Over the years, multiple studies have investigated different methods to more accurately identify CECs in order to use them as therapeutic biomarkers to confirm a diagnosis, predict the course of the disease, or support treatment decisions (**Table 1**)[130].

1.2.2.3 Hematopoietic stem cells

Blood is one of the most highly regenerative tissues, generating roughly one trillion (10^{12}) cells per day in the adult human bone marrow[131]. Hematopoiesis is the process by which the cellular components of the blood are formed during embryonic development and through the lifetime of an organism to produce and replenish the blood system[132]. The hematopoietic system is made of various populations of highly specialized cells carrying out different roles, such as oxygen transport, immune defense, and blood clotting inhibition[133]. Within the mammalian hematopoietic organization, hematopoietic stem cells (HSCs) are arguably the most well-characterized and rare cell population, sitting at the top of the hematopoietic hierarchy[134]. In adults, HSCs are described as multipotent precursors, found primarily in the bone marrow, characterized by their ability to self-renew as well as to regenerate all the different cell types that proliferate and differentiate into mature blood cells comprising the blood-forming system[135]. Although HSCs were the first adult stem cells to be described, their existence has been confirmed in other tissues, such as the heart, lungs, brain, skeletal muscle, kidney, and others[136]. HSCs were the first class of stem cells to be prospectively isolated and have become a clinical standard in the treatment of a variety of blood cell diseases, such as leukemias and autoimmune disorders[137].

The hematopoietic processes have been confirmed to be considerably altered by common cardiovascular risk factors, such as hypertension, diabetes mellitus, and hyperlipoproteinemia. Similarly, atherosclerosis, MI, and HF also induce a strong effect on hematopoiesis, which has been reported by multiple studies over the years (**Table 1**)[138].

Throughout the life of the stem cell, endothelial cells are closely associated with HSCs, from the specialized endothelial cells that give rise to HSCs, to the perivascular niche of endothelial cells that regulate HSC homeostasis[139]. In the HSC niche, HSCs are regulated by a variety of chemokines, cytokines, adhesion molecules, and other signals, promoting the colonization of HSCs, and in the steady-state of self-renewal, enhancing the proliferation, and differentiation. At the same time, endothelial cells can promote HSCs proliferation and differentiation through a paracrine mechanism, where endothelial cells maintain the survival and self-renewal of HSCs by secreting SDF-1 and binding to the hematopoietic stem cell surface receptor CXCR-4[140].

Table 1. Overview of human studies that quantified circulating levels of EPCs, CECs, and HSCs in the context of cardiovascular diseases.

Study	Subjects	Principal findings
Farinacci *et al.* (2019)[130]	HFpEF* HFrEF* DN* arterial hypertension* age-matched healthy controls*	EPCs levels were similar between healthy subjects, patients with HFrEF, and HFpEF. CEC levels were not elevated in patients with HFrEF compared to healthy subjects ↑ CEC levels in patients with HFpEF compared to healthy subjects.
Vali Shaik *et al.* (2018)[141]	CAD, *n* = 50 age-matched healthy controls, *n* = 50	↓ EPCs levels in patients with CAD compared to healthy subjects.
Regueiro *et al.* (2015)[142]	AMI or atherothrombotic stroke, *n* =150 healthy controls, *n* = 145	↑ EPCs and CECs levels in patients with AMI compared to healthy subjects. EPCs and CECs levels were not higher in stroke patients compared to healthy subjects.
Damani *et al.* (2013)[143]	STEMI, *n* = 50 healthy controls, *n* = 44	↑ CECs levels in patients with MI compared to healthy subjects.
Fortini *et al.* (2011)[144]	HF, *n* = 97 gender and age-matched healthy controls, *n* = 23	↑ EPCs and HSCs levels in patients with HF compared to healthy subjects.
Liguori *et al.* (2008)[145]	CHD, *n* = 40 healthy controls, *n* =15	↓ EPCs levels in patients with CHD and migratory capacity was significantly impaired compared to healthy subjects. ↓ HSCs levels in patients with CHD compared to healthy subjects.

AMI, acute myocardial disease; **CAD**, coronary artery disease; **CECs**, circulating endothelial cells; **CHD**, coronary heart disease; **DN**, diabetic nephropathy; **EPCs**, endothelial progenitor cells; **HF**, heart failure; **HFpEF**, heart failure with reduced ejection fraction; **HFrEF**, heart failure with preserved ejection fraction; **HSCs**, hematopoietic stem cells; **MI**, myocardial infarction; **STEMI**, ST-elevation myocardial infarction.
* The number of participants was not disclosed.

1.2.3 Endothelial dysfunction - a hallmark of cardiovascular diseases

Endothelial dysfunction is characterized by a shift in the actions of the endothelium toward reduced vasodilatation, a proinflammatory state, and prothrombic properties[146]. It is a well-established response to major cardiovascular risk factors, such as diabetes mellitus, hypertension, dyslipidemia, aging, hypercholesterolemia, and tobacco toxins[147]. As the endothelial function deteriorates, the vascular homeostasis becomes impaired resulting in a decline of the antioxidant and anti-inflammatory effects, as well as an increase of the vascular permeability to lipoproteins, an enhancement of the expression of inflammatory cytokines, and an upregulation of leukocyte adhesion molecules[98,148]. Among various complex mechanisms, oxidative stress has been shown to play a pivotal role in the pathogenesis of vascular failure, particularly vascular endothelial dysfunction mainly by a loss of local bioavailability of NO (**Figure 5**)[149].

In healthy endothelial cells, NO is considered to be the central mechanism responsible for the preservation of vascular homeostasis[150]. The impairment of NO bioavailability can be due to decreased synthesis of NO and increased oxidative degradation by reactive oxygen species (ROS)[151,152]. In this context, an increase of ROS production plays a critical role in the initiation and progression of endothelial dysfunction by promoting the upregulation of several mechanisms, such as the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, NO inactivation, the formation of peroxynitrite ($ONOO^-$), uncoupling of eNOS, and stimulation of endothelin expression[153].

The impaired endothelium-dependent vasodilatation is a well-established hallmark of endothelial dysfunction, which is a key mediator in the pathogenesis and progression of multiple cardiovascular disorders including atherosclerosis, hypercholesterolemia, stroke, CAD, and HF[105]. Altered redox state with overproduction of ROS are major features in the characterization of HF. In failing hearts, the presence of abnormal cardiac and vascular phenotypes are thought to be caused partly by imbalances between NO bioavailability and oxidative stress[103]. In the early stages of HF, inflammatory mediators from the myocardium and altered local shear forces modify gene expression, leukocyte infiltration, increased cytokine and ROS generation, and reduced NO bioavailability[154]. Nevertheless, it is difficult to determine the relationship between HF and endothelial dysfunction – which one of them is the victim, and which is the culprit[98].

Vascular oxidative stress is also responsible for promoting systemic inflammation via immune activation. Activated immune cells migrate into the vasculature and release several factors including ROS, metalloproteinases, cytokines, and chemokines promoting disruption of the endothelium and causing vascular damage by enhancing vasoconstriction and remodeling of blood vessels[155].

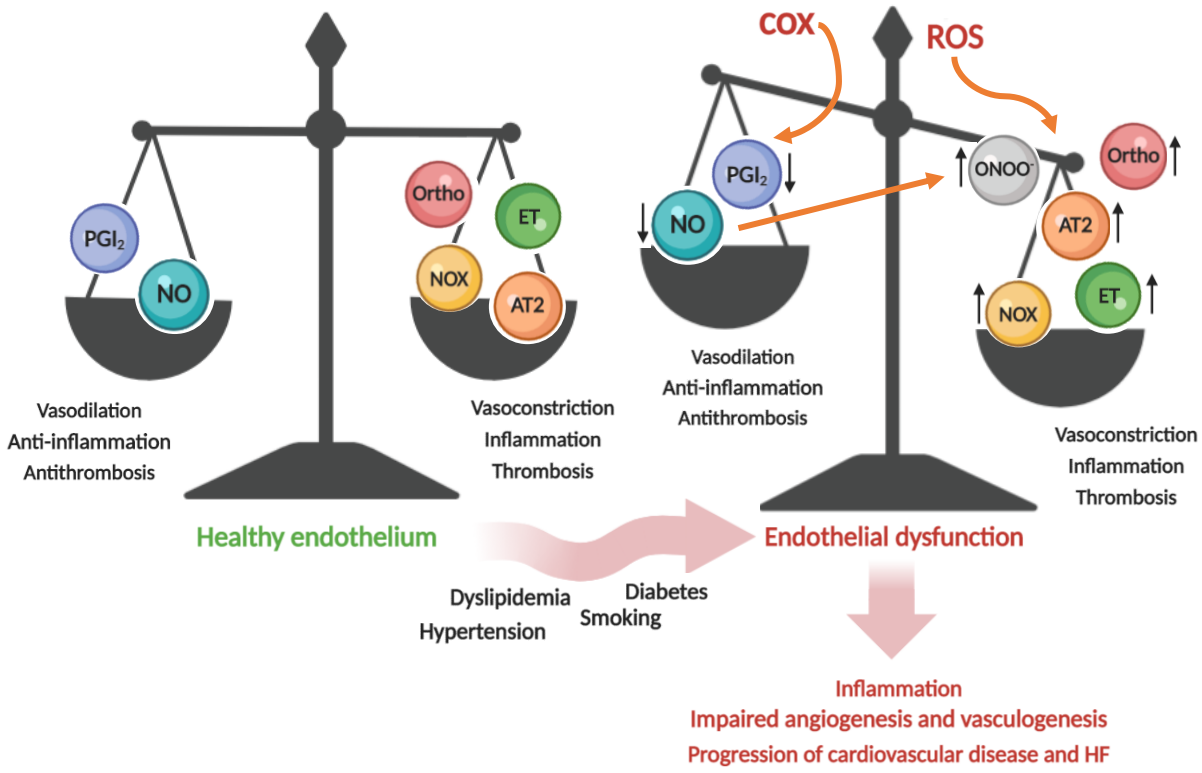

Figure 5. Pathophysiology of endothelial dysfunction. The healthy endothelium keeps a crucial balance between vasodilating, anti-inflammatory, and antithrombotic factors on one side and vasoconstricting, inflammatory, and thrombotic factors on the other. In endothelial dysfunction, increased oxidative stress triggered by comorbidities leads to a decline of the NO bioavailability through the reduction of NO to $ONOO^-$. In addition, an increase of vasoconstriction, vascular remodeling, and inflammation results in systemic inflammation, impaired angiogenesis, and reduced vasculogenesis contributing to the progression of cardiovascular disease and HF. **AT2**, angiotensin 2; **COX**, cyclooxygenase; **ET**, endothelin; **HF**, heart failure; **NO**, nitric oxide, **NOX**, nicotinamide adenine dinucleotide phosphate oxidase; **$ONOO^-$**, peroxynitrite; **Ortho**, orthosympathetic nerve activity; **PGI^2**, prostacyclin; **ROS**, reactive oxygen species. The figure was produced using Biorender. Adapted from Panth *et al.*[154] and Gevaert *et al.*[156].

CHAPTER II: OBJECTIVES

HF has been recognized as a major and growing medical and economic burden on the healthcare system due to its increasing prevalence, considerable morbidity, high mortality, and rapidly expanding health care cost[80,157]. Over the years, HF incidence has steadily increased worldwide, as traditional cardiovascular risk factors, such as diabetes, hypertension, obesity, dyslipidemia, and tobacco toxins remain a persistent problem[158]. Several of these risk factors are associated with endothelial dysfunction, which plays a key role in the pathophysiology of several cardiovascular diseases, including HF[159].

CECs are mature cells that have been shed from the lining of the vascular wall into the blood stream[128]. CECs are often present in very low numbers but are increased in a variety of cardiovascular diseases, in which they appear to be a marker of vascular dysfunction and damage[160]. In contrast, circulating EPCs arise from the bone marrow in response to tissue damage. EPCs are thought to be originated from HSCs, which gives rise to immune cells in a process called hematopoiesis. While the hematopoietic supply of inflammatory immune cells plays an important role in the development of cardiovascular diseases, cardiovascular diseases in turn strongly affects hematopoiesis[161]. Circulating levels of EPCs and CECs have been recognized as useful markers of vascular damage and endothelial repair in response to tissue injury, such as myocardia ischemia[97]. Additionally, the presence of cardiovascular risk factors can impair the number and function of CECs, EPCs, and HSCs, which may contribute to the perpetuation of the disease, the development of acute complications, and worsening of cardiac function[121,162].

Due to the factors mentioned above, the main goal of this study was to assess whether levels of circulating EPCs, CECs, and HSCs are markers of the cellular response to vascular injury in patients with HFrEF. We hypothesized that an increased number of CECs and a decreased number of EPCs would be observed in patients with HFrEF per comparison to a group of subjects presenting similar cardiovascular risk factors but without established cardiovascular disease. To achieve this goal, a combination of markers was used in peripheral blood samples in order to assess the number of circulating EPCs, CECs, and HSCs via flow cytometry analysis. We also compared the levels of circulating EPCs, CECs, and HSCs between subgroups regarding the presence of cardiovascular risk factors (e.g. diabetes mellitus) and the etiology of HF.

CHAPTER III: METHODS

3.1 Study design

A total of 82 participants, between the ages of 30 and 80 years old – 41 in each group – were enrolled in this cross-sectional study. In addition to demographic and clinical data, this study included venous blood collection for further detection of circulating EPCs, CECs, and HSCs levels by flow cytometry (**Figure 6**). All participants underwent the same assessment protocol.

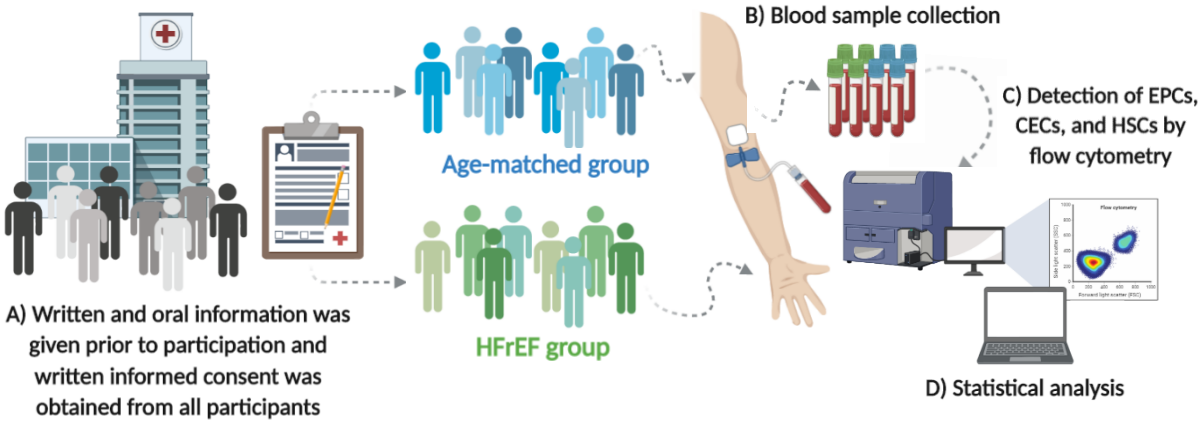

Figure 6. Study design schematization.

3.2 Participants

Eighty-two participants, between the ages of 30 and 80 years old, were recruited for this study. Participants were divided into two groups (41 in each group): patients previously diagnosed with HFrEF, followed at a central hospital located in the Porto region, Portugal; and age-matched subjects presenting similar cardiovascular risk factors but without established cardiovascular disease, followed at a primary health center located in the Aveiro region, Portugal.

The inclusion criteria for patients with HFrEF were as follows: participants were recruited according to the guidelines established by ESC[38], i.e. reduced LVEF (≤ 40%) and a NYHA functional capacity class II and III[63]. In addition, patients had to be clinically stable and receiving an optimal and stable medication regimen for the last six months prior to

inclusion in this study. Exclusion criteria included: i) unstable angina pectoris; ii) abnormal hemodynamic response; iii) uncontrolled metabolic disorders; iv) uncompensated HF; v) NYHA class IV symptoms; vi) significant valvular heart disease; vii) complex ventricular arrhythmias (at rest or during maximal exercise test); viii) severe aortic stenosis; ix) pulmonary and renal co-morbidities; x) acute myocarditis; xi) uncontrolled hypertension or orthopedic limitations that prevent physical exercise.

The inclusion criteria for the age-matched group were as follows: i) 30-80 years old; ii) free of established cardiovascular diseases (e.g., heart failure, previous myocardial infarction, stroke, coronary artery disease, among others), but presenting at least one modifiable cardiovascular risk factor (e.g. idiopathic hypertension, dyslipidemia, smoking habits, type II diabetes mellitus, and obesity). The exclusion criteria were the same as for the HFrEF group.

3.3 Ethical statement

This study was approved by the local ethics committee and all the procedures were conducted according to the principles expressed in the Declaration of Helsinki[163]. Written and oral information was given and written informed consent was obtained from all participants prior to inclusion.

3.4 Demographical and clinical characteristics

Demographic and clinical data (age, gender, and cardiovascular risk factors), as well as medication, were retrieved from clinical records and validated through a personal interview with the participants.

Anthropometric measurements, such as height and weight were determined using a stadiometer and a scale (InnerScan BC-522, Tanita, Tokyo, Japan), respectively. Body mass index (BMI) (kg/m^2) was calculated by weight (kg) divided by the square of height (meters).

Regarding cardiorespiratory fitness in patients with HFrEF, maximal oxygen uptake was measured during a maximal or symptom-limited treadmill cardiopulmonary exercise test using the modified Bruce protocol[164,165]. As for the age-matched group, maximal oxygen uptake was estimated using the Chester Step Test (CST) which was performed according to

test manual recommendations[166]. In brief, the CST can be divided into five stages, with each stage having a duration of two minutes. The step cadence was set by a tape and started at 15 steps per minute and increased by 5 steps per minute every two minutes. The test ends when 80% of the age-estimated maximal heart rate was exceeded, a value above 14 on the perceived exertion scale was reached or the participant was unable to maintain the metronome-set pace. The CST is a valid tool to assess cardiorespiratory fitness in adults presenting cardiovascular risk factors, namely hypertension[167].

Resting systolic and diastolic blood pressure and HR were assessed with the participants in a sitting position with the right arm supported and relaxed on a table in order to place the brachial artery at heart level. After a ten-minute rest, at least two blood pressure measurements were made at intervals of one minute. The average of these two measurements was recorded. Additional measurements were made only if the first two readings differed by more than 10 mmHg, which in that case, the blood pressure was recorded as the average of the last two blood pressure readings[168].

The participants were asked to avoid strenuous exercise, caffeinated products, alcohol consumption for at least 24 hours prior, and not to smoke or eat 3 hours before the evaluation.

3.5 Assessment of risk factors

Risk factors were evaluated in all participants based on their medical records. Type II diabetes mellitus (T2DM) was characterized by high blood glucose (300 – 350 mg/dL) and hemoglobin A1c (HbA1c) levels (> 10%); requiring the initiation of pharmacologic therapy, such as metformin or insulin, prescribed by a physician[169]. Hypertension was diagnosed as either a systolic or a diastolic increase in blood pressure (> 140/90 mmHg, respectively) or the use of antihypertensive therapy, such as ACE-I, angiotensin II receptor blockers (ARBs), mineralocorticoid receptor antagonists (MRAs), or a combination[168]. Dyslipidemia was defined as the co-existence of high levels of low-density lipoproteins (LDL) (≥ 100 mg/dL), low levels of high-density lipoproteins (HDL) (< 40 mg/dL in men; < 45 mg/dL in women), high levels of triglycerides (> 40 mg/dL), and elevated total serum cholesterol levels (> 190 mg/dL), or the use of lipid-lowering agents[170,171]. Obesity was defined according to the BMI cut-points, established by the WHO as follows: underweight

(BMI < 18.5 kg/m^2), normal weight (BMI between 18.5 and 24.9 kg/m^2), overweight (BMI between 25.0 and 29.9 kg/m^2), and obesity (BMI ≥30 kg/m^2)[172]. Smoking habits were assessed by two categories: active smoker or not.

3.6 Blood sample collection

Whole peripheral blood was drawn by venipuncture in the antecubital vein, collected into ethylenediaminetetraacetic acid (EDTA) coated tubes and treated with TransFix® (Cytomark, Caltag Medsystems Ltd, Buckingham, UK), according to manufacturer's instructions at a 1:5 ratio immediately after collection. Blood samples were then transported at room temperature and proceeded within 7 days. The addition of TransFix® cellular antigen stabilizing reagent was proven to stabilize blood cells, allowing to delay the analyses up to 7 days after blood collection[173].

3.7 Quantification of circulating EPCs, CECs, and HSCs by flow cytometry

Staining and analysis were performed using a protocol adapted from Ahmed *et al*[174]. For the quantitative assessment of circulating EPCs, CECs, and HSCs by flow cytometry, whole blood samples were incubated for 10 minutes with an FcR-blocking reagent in order to block unwanted binding of antibodies to human Fc receptor-expressing cells. All staining procedures were executed at room temperature. For the evaluation of circulating EPCs, CECs, and HSCs in the peripheral blood, samples were incubated with BV410 CD34 (BD Horizon), PE CD309 (VEGFR-2/KDR; BD Pharmingen), FITC CD144 (BD Pharmingen), BV510 CD45 (BD Horizon), and APC CD133/1 (Miltenyi Biotec), according to manufacturer's instructions. After erythrocyte lysis, at least 500.000 $CD45^+$ and a minimum of 100 $CD34^+$ cells were acquired on a BD FACS Canto II™ system using BD FACSDiva™ software. All samples were analyzed in duplicate. Data was analyzed using Infinicyt™ (Cytognos).

The EPCs were defined as $CD45^{low}/CD34^+/CD309^+/CD133^+/CD144^-$ cells, the CECs as $CD45^{low}/CD34^+/CD309^+/CD133^-/CD144^+$, and HSCs as $CD45^+/CD34^+/CD309^-/CD133^+/CD144^-$. EPCs, CECs, and HSCs count were expressed as percentage of leukocytes ($CD45^+$ cells). The intra-assay variation ranged from 1.7 to 7.9%. Flow cytometry gating

strategy for quantitative assessment of EPCs, CECs, and HSCs started by removing doublets using forward scatter (FSC)-Height (FSC-H) by FSC-Area (FSC-A) (R0 plot 1). Then, red blood cells, platelets, and other debris were removed from the analyses based on their FCS/ side scatter (SSC) dot-plot (R1 plot 2). Next, a gate was set on $CD45^{+}$, including $CD45^{dim}$ and $CD45^{bright}$ and excluding $CD45^{-}$ (R2 plot 3). Next, the events in gate R1 were displayed on a CD34 *versus* SSC dot plot (plot 4) and a third gate (R3) was defined in a sequential strategy in order to include the cluster of $CD34^{+}$ events (R3 plot 4). The fifth plot was obtained by plotting $CD34^{+}$ cells presenting low SSC and low CD45 fluorescence ($SSC^{low}/CD45^{dim}$ cells) were gated (R4 plot 5). Then, on a CD34 *versus* CD309 (VEGFR2/KDR) dot-plot the EPCs/CECs (R6) and HSCs (R7) were gated. Finally, on a CD144 *versus* CD133 dot-plot (plot 6), EPCs were identified as $CD309^{+}$, $CD133^{+}$, and $CD144^{-}$ (R8) and CECs as $CD309^{+}$, $CD133^{-}$, and $CD144^{+}$ (R9). The HSCs were identified on a CD144 *versus* CD133 dot-plot (plot 8) as $CD309^{-}$, $CD144^{-}$ and $CD133^{+}$ (R10).

For illustrative purposes, **figure 7** represents the flow cytometry gating strategy for the quantification of EPCs.

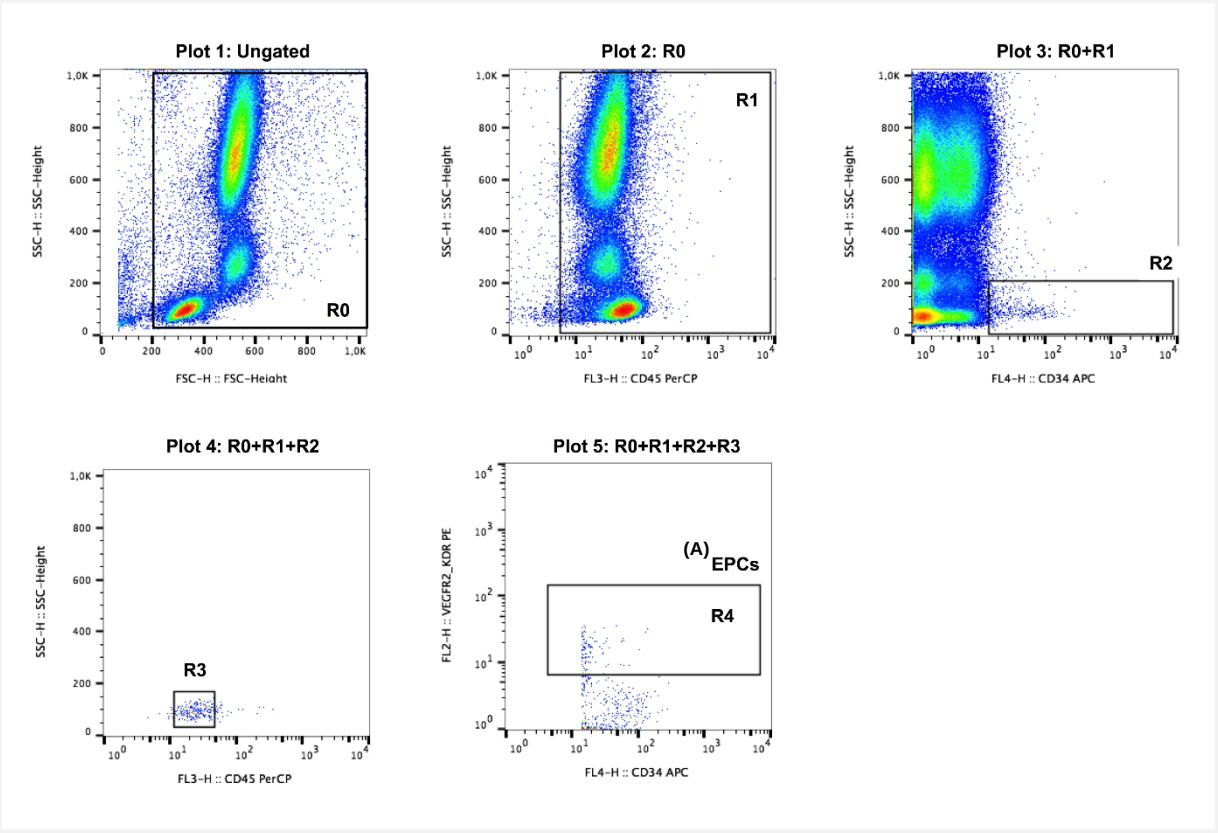

Figure 7. Flow cytometry gating strategy for the identification of EPCs. The gating strategy for quantitative assessment of EPCs, CECs, and HSCs started by removing doublets using forward scatter FSC-H by FSC-A. Then, red blood cells, platelets, and other debris were removed from the analyses based on their FCS/SSC dot-plot. Next, a gate was set on $CD45^{+}$, including $CD45^{dim}$ and $CD45^{bright}$ and excluding $CD45^{-}$. Next, the events in the first gate were displayed on a CD34 *versus* SSC dot plot and a third gate was defined in a sequential strategy in order to include the cluster of $CD34^{+}$ events. The fifth plot was obtained by plotting $CD34^{+}$ cells presenting low SSC and low CD45 fluorescence ($SSC^{low}/CD45^{dim}$ cells) were gated. Then, on a CD34 *versus* CD309 (VEGFR2/KDR) dot-plot the EPCs/CECs and HSCs were gated. Finally, on a CD144 *versus* CD133 dot-plot, EPCs were identified as $CD309^{+}$, $CD133^{+}$, and $CD144^{-}$. **EPCs**, endothelial progenitor cells; **FSC-A**, Forward Scatter-Area; **FSC-H**, Forward Scatter-Height; **SSC**, Side Scatter.

3.8 Statistical analyses

Statistical analysis was conducted using IBM SPSS Statistics Software 26 (IBM Corporation, Chicago, IL, USA).

Continuous variables were tested for normal distribution by the Shapiro-Wilk test and expressed as mean ± standard deviation or median with interquartile range for parametric and nonparametric data, respectively. Circulating EPCs, CECs, and HSCs were normally distributed after log-transformation; their means were back-transformed, and the values are presented in the original scale for clarity reasons.

For comparison of continuous variables between groups (HFrEF and age-matched group) and subgroups (e.g. HFrEF with ischemic *versus* non-ischemic etiology), an Independent *t*-test was used when variables were normally distributed (weight, BMI, diastolic blood pressure, VO_2 peak, CECs, EPCs, and HSCs), while the Mann-Whitney *U* test was used for nonparametric continuous variables (age, height, and systolic blood pressure). Comparison of categorical variables (gender, etiology, T2DM, hypertension, dyslipidemia, smoking status, BMI category, and medication) between groups was assessed by Pearson Chi-Square test or with Fisher's exact test, as appropriate. Categorical data was expressed as absolute numbers with their respective percentages.

For all analyses, a two-sided value of $\boldsymbol{P} < 0.05$ was considered statistically significant.

CHAPTER IV: RESULTS

4.1 Participants clinical characteristics

The clinical characteristics, biochemical parameters, and participant's medication are summarized in **Table 2**. A total of 82 subjects (mean age, 62.4 ± 1.3 years; male subjects, 74.4%) were enrolled in this study. The majority (65.9%, n = 54) of the 82 participants presented dyslipidemia and slightly more than half (51.2%, n = 42) had hypertension. Approximately one third (32.9%, n = 27) were diagnosed with T2DM and 21 (25.6%) were classified as obese. Regarding smoking habits, 8 (11.1%) were active smokers. The age-matched group included 41 subjects (median age, 66.0 [17.0] years; male subjects, 78.0%) with cardiovascular risk factors. Among the 41 patients with HFrEF (median age, 63.0 [15.0] years; male subjects, 70.7%), 20 patients (48.8%) had an ischemic and 21 (51.2%) a non-ischemic etiology.

With respect to medication, 37 patients were under ACE-I (45.1%), 24 under ARBs (29.3%), 24 under antidepressants (29.3%), 39 under β-blockers (47.6%), 9 under calcium channel blockers (10.9%), 38 under diuretics (46.3%), 22 under oral antidiabetic (26.8%), and 53 under statins (64.6%). In relation to the specific treatment of patients with HFrEF, 28 were under anticoagulants (63.4%), 12 under antiaggregants (29.3%), 4 under antiarrhythmics (9.8%), 5 under antianginals (12.2%), and 3 under digoxin (7.3%).

No significant differences in risk factor profiles were found when comparing the two groups in relation to hypertension and smoking status ($\boldsymbol{P} > 0.05$). HFrEF group had significantly more patients diagnosed with T2DM and dyslipidemia when compared with the age-matched group (X^2 (1) = 12.424; $\boldsymbol{P} \leq 0.001$ and X^2 (1) = 10.630; $\boldsymbol{P} = 0.001$, respectively). The peak of oxygen uptake (VO_2 peak) was significantly lower in patients with HFrEF (17.5 ± 4.5 $ml/kg^{-1}/min^{-1}$ *versus* 31.1 ± 6.9 $ml/kg^{-1}/min^{-1}$, respectively) compared to age-matched subjects (t (68) = - 9.866; $\boldsymbol{P} \leq 0.001$). Moreover, the systolic and diastolic blood pressure were significantly higher in age-matched subjects (126.0 [19.0] versus 119.0 [24.0] mmHg and 78.1 ± 1.4 *versus* 71.3 ± 1.7 mmHg, respectively) compared to patients with HFrEF ((U = 1140; $\boldsymbol{P}$ = 0.005) and (t (80) = - 3.032; $\boldsymbol{P}$ = 0.003), respectively) (**Table 2**).

Table 2. Comparison of the clinical characteristics between HFrEF patients and subjects with cardiovascular risk factors (age-matched group).

Variables	HFrEF Group	Age-matched Group	*P*-value
N	41	41	-
Age (years)	63.0 [15.0]	66.0 [17.0]	0.597
Gender, male, *n* (%)	29 (70.7)	32 (78.0)	0.448
Height (m)	1.7 [0.1]	1.6 [0.1]	≤ 0.001
Weight (kg) [a)]	72.0 ± 2.3	67.4 ± 1.8	0.127
BMI (Kg/m^2) [a)]	26.6 ± 0.8	27.3 ± 0.6	0.487
LVEF (%)	38.3 ± 1.8	-	-
VO_2 peak ($ml/kg^{-1}/min^{-1}$) [a)]	17.5 ± 0.8	31.1 ± 1.2	≤ 0.001
Blood pressure (mmHg)			
Systolic	119.0 [24.0]	126.0 [19.0]	0.005
Diastolic [a)]	71.3 ± 1.7	78.1 ± 1.4	0.003
Etiology, *n* (%)			
Ischemic	20 (48.8)	-	-
Non - ischemic	21 (51.2)	-	-
Cardiovascular risk factors, *n* (%)			
Type II diabetes mellitus	21 (51.2)	6 (14.6)	≤ 0.001
Hypertension	23 (56.1)	19 (46.3)	0.377
Dyslipidemia	34 (82.9)	20 (48.8)	0.001
Smoking status, *n* (%)			0.071
Yes	7 (17.5)	2 (4.9)	
No	33 (82.5)	39 (95.1)	
BMI category, *n* (%)			0.410
Underweight	2 (4.8)	0	
Normal range	15 (35.7)	12 (29.3)	
Overweight (pre-obese)	14 (34.2)	18 (43.9)	
Obese	10 (24.4)	11 (26.8)	

Variables	HFrEF Group	Age-matched Group	*P*-value
Medication, *n* (%)			
ACEIs	26 (63.4)	11 (26.8)	≤ 0.001
ARBs	19 (41.3)	5 (12.2)	≤ 0.001
Anticoagulants	28 (63.3)	0	≤ 0.001
Antiaggregants	12 (29.3)	0	≤ 0.001
Antiarrhythmics	4 (9.8)	0	0.116
Antianginals	5 (12.2)	0	0.055
Antidepressants	15 (36.6)	9 (22.0)	0.145
β-blockers	33 (80.5)	6 (14.6)	≤ 0.001
CCBs	3 (7.3)	6 (14.6)	0.289
Digoxin	3 (7.3)	0	0.241
Diuretics	32 (78.0)	6 (14.6)	≤ 0.001
Oral antidiabetic	16 (39.0)	6 (14.6)	0.013
Statins	34 (82.9)	19 (41.3)	≤ 0.001

ACEI, angiotensin-converting enzyme inhibitor; **ARB**, angiotensin II receptor blocker; **BMI**, body mass index; **CCB**, calcium channel blocker; **LVEF**, left ventricular ejection fraction; **VO_2 peak**, peak of oxygen uptake.

Results are expressed as mean ± SD, median [interquartile range], or as number (%).

[a)] Independent sample *t*-test. Equal variances assumed:
(Levene's test for equality of variables: $\boldsymbol{P} > 0.05$).

[b)] Independent sample *t*-test. Equal variances not assumed:
(Levene's test for equality of variables: $\boldsymbol{P} < 0.05$).

4.2 Circulating EPCs, CECs, and HSCs levels between patients with HFrEF and age-matched subjects

Regarding levels of circulating EPCs and CECs, a significant difference between patients with HFrEF (n = 41) and age-matched subjects (n = 41) was observed ($\boldsymbol{P} < 0.05$). Patients with HFrEF presented lower levels of circulating EPCs ($5.28 \times 10^{-3} \pm 6.83 \times 10^{-4}$ % *versus* $7.76 \times 10^{-3} \pm 4.91 \times 10^{-4}$ %) compared to subjects with cardiovascular risk factors (t (62.2) = - 4.630; $\boldsymbol{P} \leq 0.001$) (**Figure 8A**). Furthermore, patients with HFrEF had CECs levels reduced by 22% ($5.11 \times 10^{-3} \pm 7.87 \times 10^{-4}$ % *versus* $6.51 \times 10^{-3} \pm 5.21 \times 10^{-4}$ %) when compared to subjects with cardiovascular risk factors (t (70.4) = - 2.937; $\boldsymbol{P} = 0.005$) (**Figure 8B**). Lastly, levels of HSCs were not significantly different between patients with HFrEF and subjects with cardiovascular risk factors ($1.72 \times 10^{-2} \pm 1.22 \times 10^{-3}$ % *versus* $1.68 \times 10^{-2} \pm 7.84 \times 10^{-4}$ %; $\boldsymbol{P} = 0.590$) (**Figure 8C**).

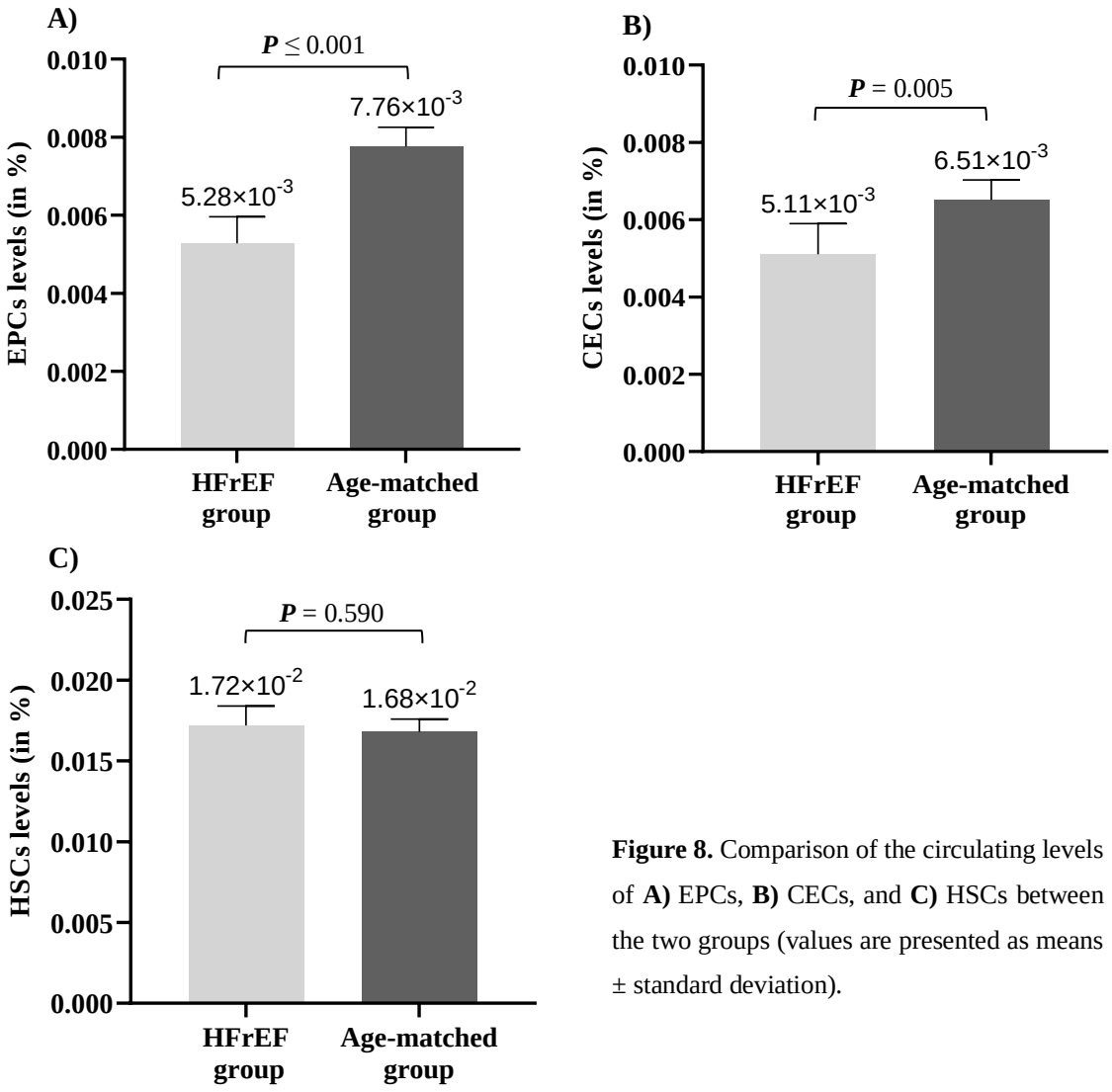

Figure 8. Comparison of the circulating levels of **A)** EPCs, **B)** CECs, and **C)** HSCs between the two groups (values are presented as means ± standard deviation).

4.3 Impact of clinical factors on EPCs, CECs, and HSCs levels in patients with HFrEF

4.3.1 Circulating EPCs, CECs, and HSCs levels in patients with ischemic and non-ischemic etiology

There were no statistically significant differences in levels of circulating EPCs, CECs, and HSCs between patients with ischemic (n = 20) and non-ischemic HF (n = 21) (***P*** > 0.05) (**Figure 9**). Nevertheless, CECs tended to circulate in lower number in patients with non-ischemic etiology (3.61 x 10^{-3} ± 2.71 x 10^{-3} % *versus* 6.69 x 10^{-3} ± 6.38 x 10^{-3} %) compared to patients with ischemic HF (***P*** = 0.057) (**Figure 9B**).

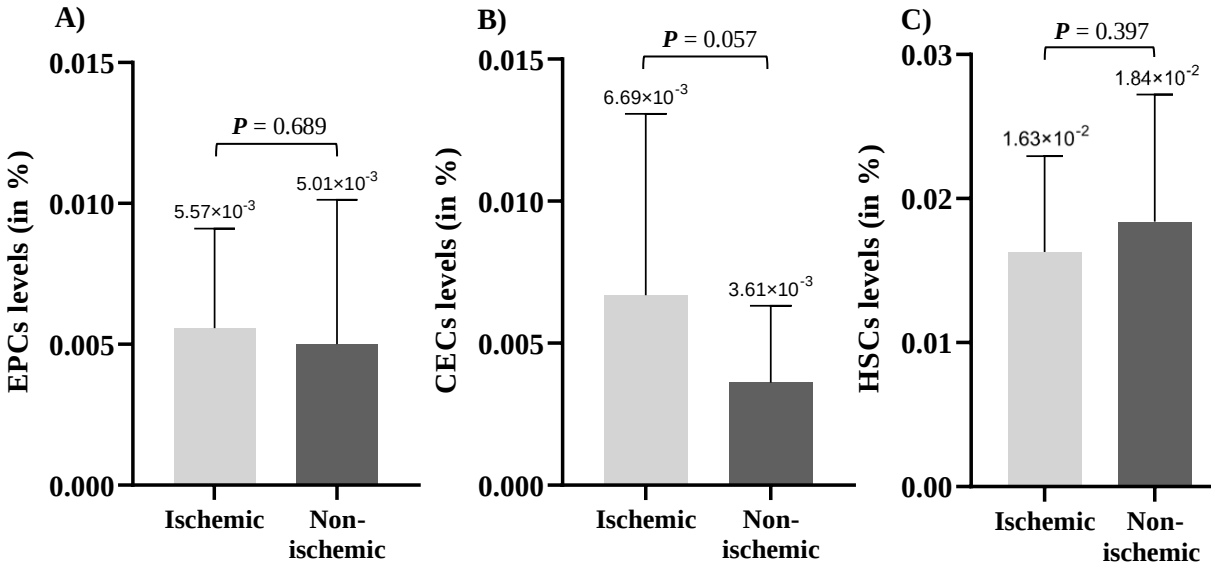

Figure 9. Comparison of the circulating levels of **A)** EPCs, **B)** CECs, and **C)** HSCs between patients with ischemic and non-ischemic HF (values are presented as means ± standard deviation).

4.3.2 Circulating EPCs, CECs, and HSCs levels in patients with and without T2DM

There were no statistically significant differences in circulating levels of EPCs, CECs, and HSCs between patients with T2DM (n = 21) and without T2DM (n = 20) (***P*** > 0.05) (**Figure 10**). However, HFrEF patients with T2DM showed a tendency to have lower levels of CECs ($4.75 \times 10^{-3} \pm 5.38 \times 10^{-3}$ % *versus* $5.49 \times 10^{-3} \pm 4.76 \times 10^{-3}$ %) when compared to patients without T2DM (***P*** = 0.096) (**Figure 10B**).

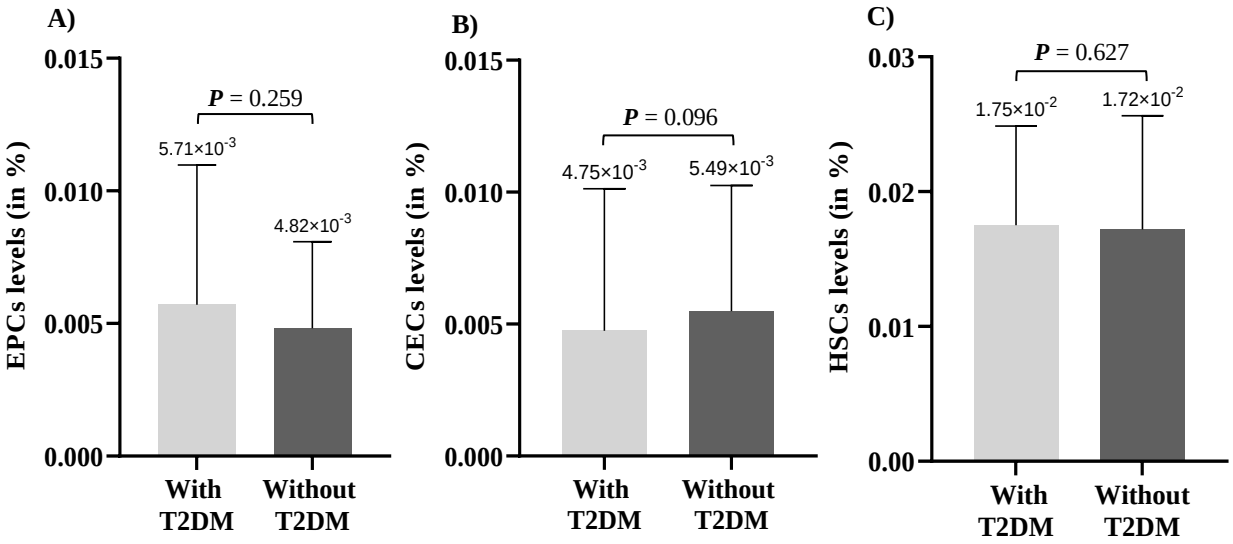

Figure 10. Comparison of the circulating levels of **A)** EPCs, **B)** CECs, and **C)** HSCs between patients with and without T2DM (values are presented as means ± standard deviation).

4.3.3 Circulating EPCs, CECs, and HSCs levels in patients diagnosed as overweight/obese and patients with a normal weight

As shown in **Figure 11A**, the number of circulating EPCs was significantly higher in patients diagnosed as overweight/obese (n = 24) ($6.10 \times 10^{-3} \pm 4.78 \times 10^{-3}$ % *versus* $4.13 \times 10^{-3} \pm 3.55 \times 10^{-3}$ %) compared to patients with a normal weight (n = 17) (t (39) = - 2.093; ***P*** = 0.043). Additionally, CECs levels were significantly higher in patients diagnosed as overweight/obese ($6.27 \times 10^{-3} \pm 5.66 \times 10^{-3}$ % *versus* $3.47 \times 10^{-3} \pm 3.54 \times 10^{-3}$ %) compared to patients with a normal weight (t (39) = - 2.447; ***P*** = 0.019) (**Figure 11B**). Lastly, there were no statistically significant differences in levels of HSCs between patients diagnosed as overweight/obese and patients with a normal weight ($1.82 \times 10^{-2} \pm 8.28 \times 10^{-3}$ % *versus* $1.61 \times 10^{-2} \pm 7.15 \times 10^{-3}$ %; ***P*** = 0.538) (**Figure 11C**).

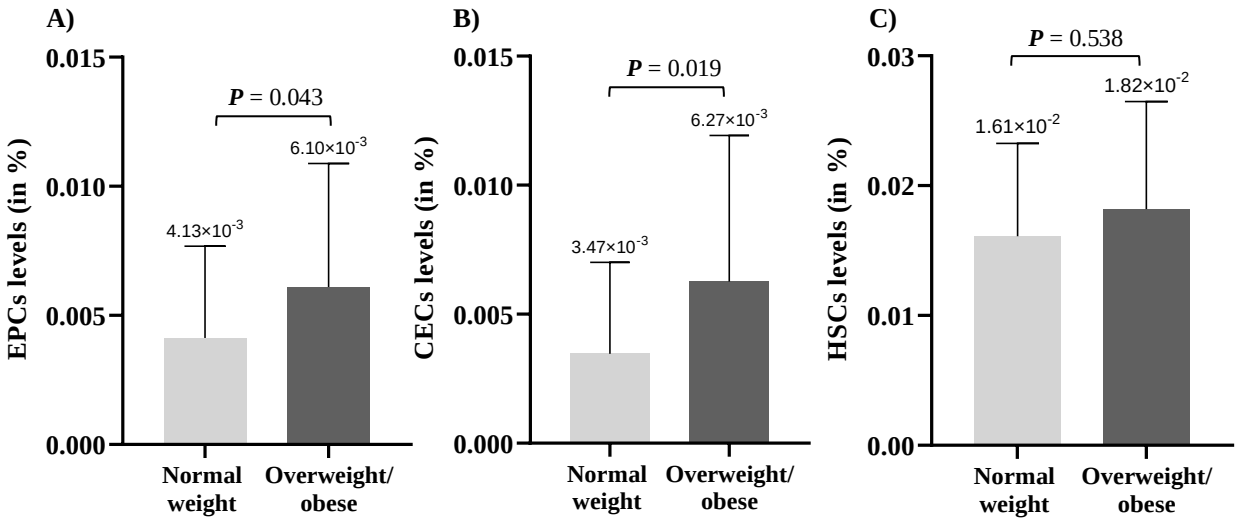

Figure 11. Comparison of the circulating levels of **A)** EPCs, **B)** CECs, and **C)** HSCs between patients with a normal weight and patients diagnosed as overweight/obese (values are presented as means ± standard deviation).

4.3.4 Circulating EPCs, CECs, and HSCs levels in patients with and without hypertension

There were no statistically significant differences in circulating EPCs, CECs, and HSCs levels between patients diagnosed with hypertension (n = 23) and without hypertension (n = 18) (***P*** > 0.05) (**Figure 12**).

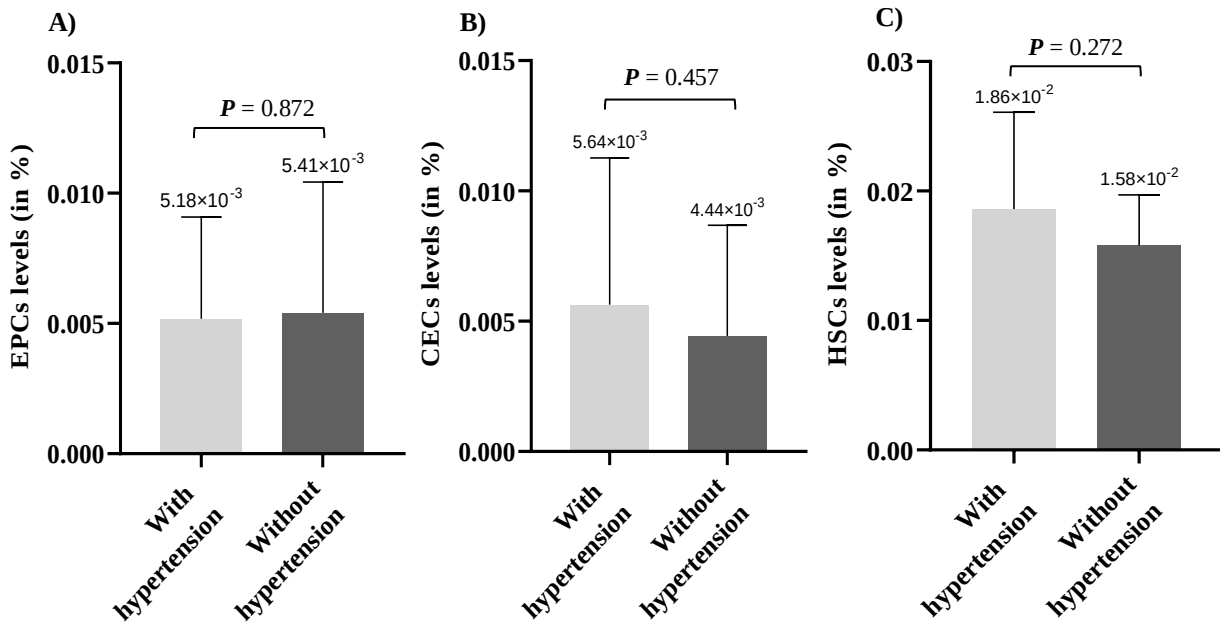

Figure 12. Comparison of the circulating levels of **A)** EPCs, **B)** CECs, and **C)** HSCs between patients with and without hypertension (values are presented as means ± standard deviation).

4.4 Circulating EPCs, CECs, and HSCs levels in age-matched subjects

4.4.1 Circulating EPCs, CECs, and HSCs levels in subjects with and without dyslipidemia

Circulating EPCs and HSCs levels were not statistically different between subjects with (n = 20) and without dyslipidemia (n = 21) (***P*** > 0.05) (**Figure 13A**, **13C**). However, the number of CECs was significantly higher in subjects with dyslipidemia ($7.74 \times 10^{-3} \pm 3.64 \times 10^{-3}$ % *versus* $5.34 \times 10^{-3} \pm 2.59 \times 10^{-3}$ %) compared to subjects without dyslipidemia (*t* (39) = - 2.105; ***P*** = 0.042) (**Figure 13B)**.

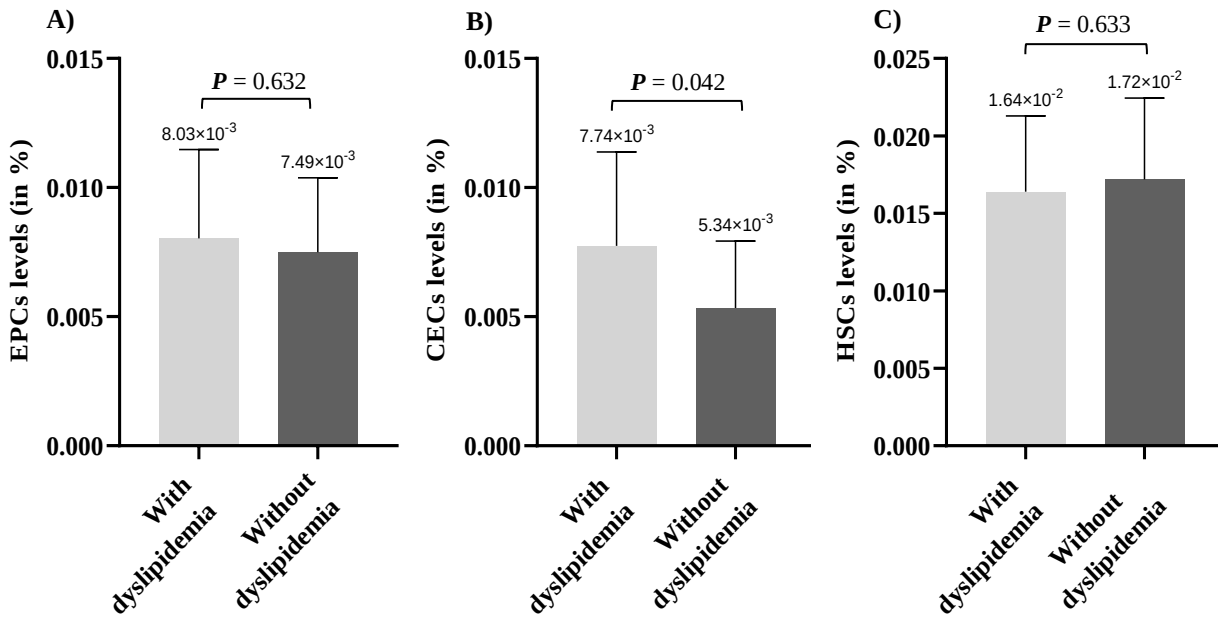

Figure 13. Comparison of the circulating levels of **A)** EPCs, **B)** CECs, and **C)** HSCs between subjects with and without dyslipidemia (values are presented as means ± standard deviation).

4.4.2 Circulating EPCs, CECs, and HSCs levels in subjects with and without hypertension

As shown in **Figure 14A**, the number of circulating EPCs was significantly higher in subjects without hypertension (n = 22) ($8.74 \times 10^{-3} \pm 3.62 \times 10^{-3}$ *versus* $6.61 \times 10^{-3} \pm 2.02 \times 10^{-3}$ %) compared to subjects with hypertension (n = 19) (t (39) = 2.377; $\boldsymbol{P}$ = 0.023). However, CECs and HSCs levels were not statistically different between subjects with and without hypertension ($\boldsymbol{P} < 0.05$) (**Figure 14B**, **14C**).

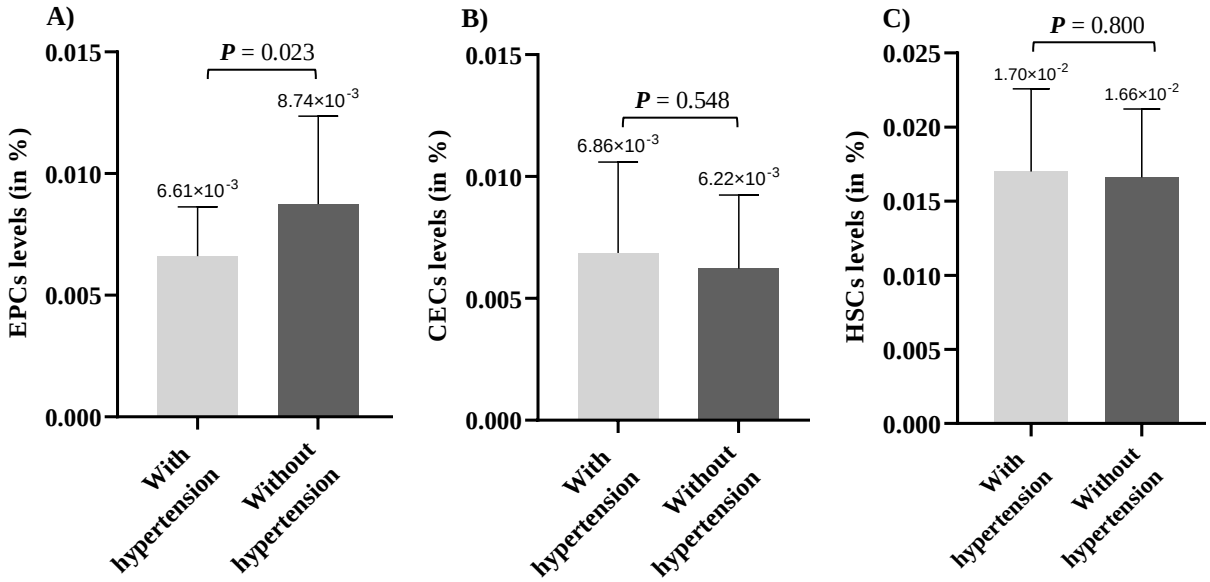

Figure 14. Comparison of the circulating levels of **A)** EPCs, **B)** CECs, and **C)** HSCs between subjects with and without hypertension (values are presented as means ± standard deviation).

4.5 Subgroup comparisons between patients with HFrEF and age-matched subjects

4.5.1 Comparison of EPCs, CECs, and HSCs levels between patients and age-matched subjects with hypertension

Circulating EPCs levels were significantly higher in age-matched subjects with hypertension (n = 19) ($6.61 \times 10^{-3} \pm 2.02 \times 10^{-3}$ % *versus* $5.18 \times 10^{-3} \pm 3.91 \times 10^{-3}$ %) compared to HFrEF patients with hypertension (n = 23) (t (33.1) = - 2.848; $\boldsymbol{P}$ = 0.008) (**Figure 15A**). Moreover, there were no statistically significant differences in levels of CECs and HSCs between patients with HFrEF and age-matched subjects with hypertension ($\boldsymbol{P}$ > 0.05) (**Figure 15B**, **15C**).

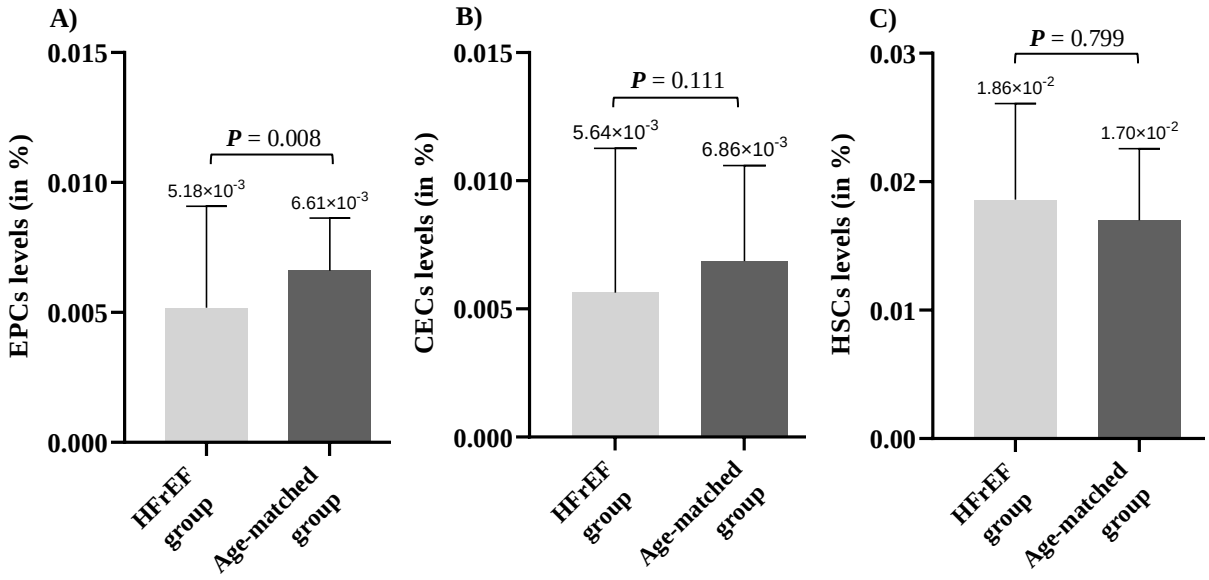

Figure 15. Comparison of the circulating levels of **A)** EPCs, **B)** CECs, and **C)** HSCs between patients with HFrEF and hypertension and age-matched subjects with hypertension (values are presented as means ± standard deviation).

4.5.2 Comparison of EPCs, CECs, and HSCs levels between patients and age-matched subjects diagnosed as overweight/obese

Circulating EPCs levels were significantly higher in age-matched subjects diagnosed as overweight/obese (n = 29) ($7.77 \times 10^{-3} \pm 3.33 \times 10^{-3}$ % *versus* $6.10 \times 10^{-3} \pm 4.78 \times 10^{-3}$ %) compared to patients with HFrEF diagnosed as overweight/obese (n = 24) (t (51) = - 2.650; ***P*** = 0.011) (**Figure 16A**). However, there were no statistically significant differences in levels of CECs and HSCs between patients with HFrEF diagnosed as overweight/obese and age-matched subjects also diagnosed as overweight/obese (***P*** > 0.05) (**Figure 16B**, **16C**).

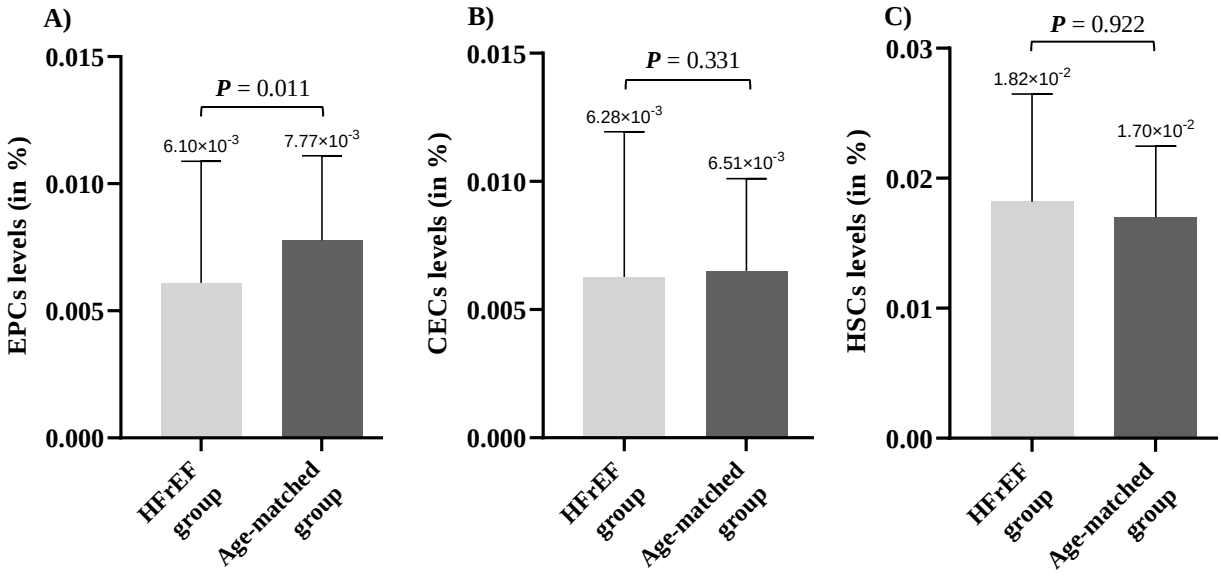

Figure 16. Comparison of the circulating levels of **A)** EPCs, **B)** CECs, and **C)** HSCs between patients with HFrEF diagnosed as overweight/obese and age-matched subjects diagnosed as overweight/obese (values are presented as means ± standard deviation).

4.5.3 Comparison of EPCs, CECs, and HSCs levels between patients and age-matched subjects diagnosed with dyslipidemia

Circulating EPCs levels were significantly higher in age-matched subjects diagnosed with dyslipidemia (n = 20) ($8.03 \times 10^{-3} \pm 3.44 \times 10^{-3}$ % *versus* $4.98 \times 10^{-3} \pm 4.02 \times 10^{-3}$ %) compared to patients with HFrEF diagnosed with dyslipidemia (n = 34) (t (52) = - 4.414; $\boldsymbol{P} \leq 0.001$) (**Figure 17A**). Moreover, CECs were significantly higher in age-matched subjects diagnosed with dyslipidemia ($7.74 \times 10^{-3} \pm 3.64 \times 10^{-3}$ % *versus* $5.19 \times 10^{-3} \pm 5.43 \times 10^{-3}$ %) compared to patients with HFrEF diagnosed with dyslipidemia (t (51) = - 3.360; $\boldsymbol{P} = 0.001$) (**Figure 17B**). No statistically significant differences were observed in HSCs levels ($\boldsymbol{P} > 0.05$) (**Figure 17C**).

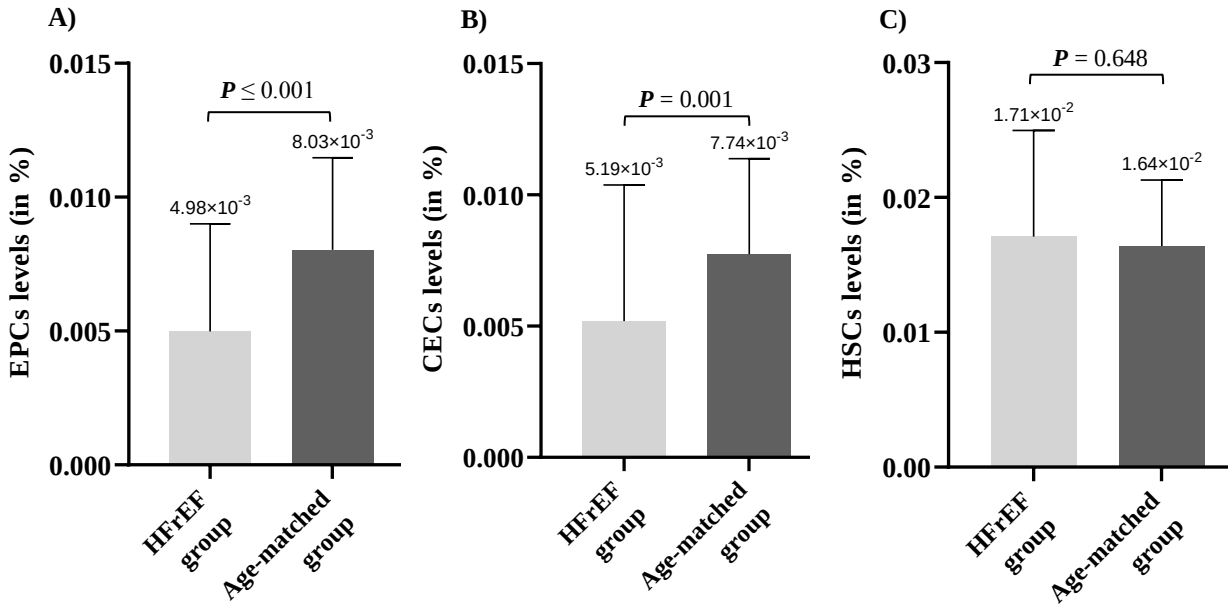

Figure 17. Comparison of the circulating levels of **A)** EPCs, **B)** CECs, and **C)** HSCs between patients with HFrEF and dyslipidemia and age-matched subjects with dyslipidemia (values are presented as means ± standard deviation).

A summary of all the results obtained in this study is shown in **Table 3**.

Table 3. Overview of the results obtained in this study

EPCs levels	CECs levels	HSCs levels
Patients with HFrEF *vs* subjects with cardiovascular risk factors		
↓ in patients with HFrEF ($5.28 \times 10^{-3} \pm 6.83 \times 10^{-4}$ % *vs* $7.76 \times 10^{-3} \pm 4.91 \times 10^{-4}$ %) (***P*** ≤ 0.001)	↓ in patients with HFrEF ($5.11 \times 10^{-3} \pm 7.87 \times 10^{-4}$ % *vs* $6.51 \times 10^{-3} \pm 5.21 \times 10^{-4}$ %) (***P*** = 0.005)	Not significantly different
Patients with HFrEF: ischemic *vs* non-ischemic etiology		
Not significantly different	Tended to be ↓ in patients with non-ischemic etiology ($3.61 \times 10^{-3} \pm 2.71 \times 10^{-3}$ % *vs* $6.69 \times 10^{-3} \pm 6.38 \times 10^{-3}$ %) (***P*** = 0.057)	Not significantly different
Patients with HFrEF: T2DM *vs* without T2DM		
Not significantly different	Tended to be ↓ in patients with T2DM ($4.75 \times 10^{-3} \pm 5.38 \times 10^{-3}$ % *vs* $5.49 \times 10^{-3} \pm 4.76 \times 10^{-3}$ %) (***P*** = 0.096)	Not significantly different
Patients with HFrEF: overweight/obese *vs* normal weight		
↑ in patients diagnosed as overweight/obese ($6.10 \times 10^{-3} \pm 4.78 \times 10^{-3}$ % *vs* $4.13 \times 10^{-3} \pm 3.55 \times 10^{-3}$ %) (***P*** = 0.043)	↑ in patients diagnosed as overweight/obese ($6.27 \times 10^{-3} \pm 5.66 \times 10^{-3}$ % *vs* $3.47 \times 10^{-3} \pm 3.54 \times 10^{-3}$ %) (***P*** = 0.019)	Not significantly different
Patients with HFrEF: hypertension *vs* without hypertension		
No statistically significant differences		
Age-matched subjects: dyslipidemia *vs* without dyslipidemia		
Not significantly different	↑ in subjects with dyslipidemia ($7.74 \times 10^{-3} \pm 3.64 \times 10^{-3}$ % *vs* $5.34 \times 10^{-3} \pm 2.59 \times 10^{-3}$ %) (***P*** = 0.042)	Not significantly different

EPCs levels	CECs levels	HSCs levels
Age-matched subjects: hypertension *vs* without hypertension		
↑ in subjects without hypertension ($8.74 \times 10^{-3} \pm 3.62 \times 10^{-3}$ *vs* $6.61 \times 10^{-3} \pm 2.02 \times 10^{-3}$ %) (***P*** = 0.023)	Not significantly different	Not significantly different
Subgroup: patients with HFrEF/hypertension *vs* subjects with hypertension		
↑ in age-matched subjects with hypertension ($6.61 \times 10^{-3} \pm 2.02 \times 10^{-3}$ % *vs* $5.18 \times 10^{-3} \pm 3.91 \times 10^{-3}$ %) (***P*** = 0.008)	Not significantly different	Not significantly different
Subgroup: patients with HFrEF/obesity/overweight *vs* subjects diagnosed with overweight/obesity		
↑ in age-matched subjects diagnosed as overweight/obese ($7.77 \times 10^{-3} \pm 3.33 \times 10^{-3}$ % *vs* $6.10 \times 10^{-3} \pm 4.78 \times 10^{-3}$ %) (***P*** = 0.011)	Not significantly different	Not significantly different
Subgroup: patients with HFrEF/dyslipidemia *vs* subjects with dyslipidemia		
↑ in age-matched subjects with dyslipidemia ($8.03 \times 10^{-3} \pm 3.44 \times 10^{-3}$ % *vs* $4.98 \times 10^{-3} \pm 4.02 \times 10^{-3}$ %) (***P*** ≤ 0.001)	↑ in age-matched subjects with dyslipidemia ($7.74 \times 10^{-3} \pm 3.64 \times 10^{-3}$ % vs $5.19 \times 10^{-3} \pm 5.43 \times 10^{-3}$ %) (***P*** = 0.001)	Not significantly different

CECs, circulating endothelial cells; **EPCs**, endothelial progenitor cells; **HFrEF**, heart failure with preserved ejection fraction; **HSCs**, hematopoietic stem cells; **T2DM**, type two diabetes mellitus.

CHAPTER V: DISCUSSION

This study was designed to assess whether circulating levels of EPCs, CECs, and HSCs were markers of vascular damage in patients with HFrEF. We hypothesized that increased numbers of CECs and decreased numbers of EPCs would be observed in patients with HFrEF per comparison to a group of subjects presenting similar cardiovascular risk factors but without established cardiovascular disease (age-matched group). There were five major findings in the present study, which can be summarized as follows. First, we observed that patients with HFrEF had significantly decreased levels of circulating EPCs and CECs compared to subjects with cardiovascular risk factors. Second, levels of HSCs were not significantly different between patients with HFrEF and subjects from the age-matched group. Third, CECs tended to circulate in a higher number in patients with ischemic HF compared to patients with non-ischemic HF. Fourth, patients with HFrEF and diagnosed as overweight/obese had significantly higher levels of circulating EPCs and CECs when compared to patients with HFrEF presenting a normal weight. Fifth, when comparing subjects from the age-matched group, subjects with dyslipidemia had significantly higher levels of CECs compared to subjects without dyslipidemia.

Over the last two decades, several researchers have focused their attention on the key role of endothelial dysfunction in the pathogenesis and progression of HF[103,159,175,176]. Regarding HFrEF, several underlying mechanisms have been pointed out as being responsible for inducing endothelial dysfunction, such as neurohormonal activation, altered shear stress, increased oxidative stress, and decline of NO production[158]. The imbalance of NO and oxidative stress can lead to impairment of the coronary endothelium-dependent vasodilator capacity, which decreases the myocardial perfusion, reduces coronary blood flow, and deteriorates ventricular function. Thus, triggering a decline of NO bioavailability and worsening of endothelial dysfunction, leading to progression of chronic HFrEF[177].

The reduced number of circulating EPCs and the simultaneous increase in CECs levels, among a large spectrum of cardiovascular diseases and risk factors, has been largely described in the current literature, suggesting their potential use as diagnostic biomarkers for endothelial dysfunction and vascular damage, as well as for the development of novel therapeutic strategies[178,179].

In the present study, we observed that patients with HFrEF had a significantly lower number of circulating EPCs when compared to subjects with cardiovascular risk factors. This finding was consistent with previously published reports in patients with HF[180]. For instance, Valgimi *et al.* were the first group of researchers to evaluate the role of circulating EPCs in patients with HF. They observed that patients with more advanced stages of congestive HF, indicated by higher NYHA functional classes (NYHA III and IV) and increased NT-proBNP levels, presented a lower number of circulating EPCs when compared with healthy subjects[181]. Furthermore, Nonaka-Sarukawa and colleagues observed that the number of EPCs, measured by the number of cells expressing $CD34^+$, was significantly lower in patients with severe congestive HF[182]. A similar study done by Huang *et al.* also found that patients diagnosed with congestive HF had a significantly lower number of circulating EPCs compared to healthy subjects[183]. In 2013, Chiang *et al.* observed that patients with HFrEF, as well as patients with HFpEF, had significantly lower levels of circulating EPCs, as well as enhanced systemic inflammation and higher NT-proBNP levels when compared with age-, gender-matched subjects with several cardiovascular risk factors, but without established HF[113]. Regarding the HF phenotype, a study by Berezin *et al.* further exhibited that EPCs levels, measured by the number of cells expressing $CD14^+$ and $CD309^+$, were significantly higher in patients with HFpEF compared to HFrEF. However, when comparing levels of $CD14/CD309^+$ cells between healthy volunteers and patients with HF, healthy volunteers presented higher levels of $CD14/CD309^+$ cells compared to patients with HF, independent of the etiology[179]. The decline of circulating EPCs levels in patients with HF can be justified by the excessive synthesis of ROS. In a state of oxidative stress, the production of ROS exceeds the ability of endogenous oxygen radicals scavenging enzyme systems, causing a reduction in NO, a substance produced by endothelial cells through eNOS activation. Reduced NO bioavailability affects both EPCs mobilization and migration from the bone marrow, disabling endothelial repair and regeneration, contributing to the progression of HF[103,119,184]. That being said, we could hypothesize that the decline of the circulating levels of EPCs observed in patients with HFrEF may be related to increased oxidative stress and endothelial dysfunction.

Endothelial cells are crucial constituents of blood vessels and play a key role in cardiovascular homeostasis[148]. Mature endothelial cells contribute to the repair of endothelial injury, however, they possess a limited intrinsic capacity to do so[185]. CECs have

been established as a reliable marker of vascular injury and damage, as high cell counts have been observed in a variety of cardiovascular diseases with wide-spread vascular damage, such as acute MI and HF[160].

Contrary to our expectations, and in opposition to most studies found in the literature, patients with HFrEF showed a significantly lower percentage of CECs compared to subjects with cardiovascular risk factors. For instance, Chong *et al.* demonstrated that CECs levels were increased in patients with acute HF when compared to healthy controls, with no significant differences between acute and chronic HF. In addition, there was also a strong correlation between CECs levels and plasma markers of endothelial injury (von Willebrand factor (vWf) and soluble E-selectin)[128,186]. Similarly, Martínez-Sales and colleagues observed that levels of CECs, as well as vWF, VEGF, and soluble E-selectin were significantly higher in patients with HF compared to healthy subjects. Furthermore, in opposition to Chong *et al*. results, levels of CECs, vWf, and VEGF were significantly higher in patients in the acute phase than in the stable phase of HF[187]. It is important to note that more recently, Farinacci *et al.* quantified levels of CECs and EPCs in patients with HFrEF, HFpEF, and age-matched healthy subjects reporting similar results to those observed in our study. More specifically, in patients with HFrEF levels of CECs were not elevated compared to healthy subjects[130]. The discrepancies reported between our result regarding CECs levels and previous studies could be due to differences in the study design (sample size, inclusion/exclusion criteria, the methodology for detection and quantification of CECs, and monoclonal antibodies utilized). For instance, in the studies performed by Martínez-Sales *et al.* and Chong *et al.*, the detection and enumeration of CECs was done by immunomagnetic isolation. However, Farinacci *et al.* used the same methodology for the quantification of CECs and the same panel of mononuclear antibodies applied in this study, obtaining similar results to ours.

Subjects from the age-matched group presented higher levels of circulating EPCs and CECs when compared to patients from the HFrEF group, which may lead us to believe that these patients have lower endothelial activation. One possible explanation for this result is the fact that patients with HFrEF may benefit from a far more controlled medical treatment compared to subjects with cardiovascular risk factors. For example, according to the PHYSA study carried out by the Portuguese Society of Hypertension, only 42.5% of the patients receiving treatment for hypertension had controlled blood pressure (< 140/90 mmHg)[188].

HSCs are responsible for the constant renewal of all blood cell types and are located in a specialized bone marrow microenvironment referred to as the HSC niche[138]. Endothelial cells are closely associated with HSCs throughout the life of the stem cell, from the specialized endothelial cells implicated in the development of HSCs, to the perivascular stem cell niche that regulates HSC homeostasis[139]. Additionally, preclinical studies have also revealed that HSCs and EPCs can enhance angiogenesis, induce vasculogenesis, improve endothelial function, and promote vascular repair[189].

Regarding HSC levels, patients with HFrEF presented similar numbers compared to age-matched subjects. A study done by Fortini *et al.* investigated the blood levels of different sub-classes of steam cells and tissue-committed stem cells in patients affected by different degrees of HF. As for levels of HSCs, patients with HF had a significantly increased number compared to healthy subjects, however, an association between higher HSCs levels and NYHA functional could not be demonstrated[144]. Furthermore, Sager *et al.* focused on exploring the number, origin, phenotype, and function of cardiac macrophages present in the non-ischemic myocardium of mice with HFrEF. They observed that HSCs recruitment and proliferation were much more accentuated during HFrEF than in steady-state. Mice with HFrEF had higher levels of bone marrow noradrenaline, which signals to hematopoietic niche cells through the β_3 adrenergic receptor, leading to higher systemic numbers of innate immune cells[190]. Another study done by Wojciech and colleagues assessed the dynamics and scale of the mobilization of HSC-enriched populations into the peripheral blood in relation to inflammatory and hematopoietic cytokines in patients with ST-segment-elevation acute myocardial infarction (STEMI). Patients with STEMI had a significantly higher number of early stem/muscle progenitor cells (expressing surface antigens CD34, CD117, CXCR4) compared to patients with stable angina and healthy subjects; with maximum cell efflux within the first hours of admission and without further significant increase after 24 hours, as well as 7 days later[191,192]. After MI, the myocardium surrounding the scared myocardial tissue undergoes intense remodeling which is critical for the development of ischemic HFrEF. MI and post-MI HF are accompanied by the release of several inflammatory mediators, such as IL-1, IL-6, TNF, CCL2, and GM-CSF, that act on their corresponding receptors on hematopoietic and niche cells in the bone marrow, promoting hematopoiesis and leukocyte release[161]. The infarcted myocardium also releases DMAPs (damage-associated molecular patterns) and ATP, responsible for the activation of toll-like receptors

on hematopoietic stem/progenitor cells (HSPCs), thereby triggering the production of innate immune cells. Such external alarm signals activate intracellular signal cascades leading to the production of several transcription factors, including C/EBPα, Egr-1, lrf8, Klf4, Mafb, and PU.1 which stimulates HSC proliferation[138].

When comparing EPCs, CECs, and HSCs levels between patients with ischemic and non-ischemic HF our results shown that CECs tended to circulate in a higher number in patients with ischemic HF. These results are in agreement with those previously reported in the literature. Increased CECs number has been described in multiple diseases of inflammatory, infectious, or ischemic origin[193]. Nadar *et al.* observed that patients with an acute ischemic stroke had a significantly higher number of CECs and markers of endothelial perturbation (e.g. plasma vWf and soluble E-selectin) when compared to healthy subjects[194]. A similar study done by Lee and colleagues concluded that an increased number of CECs, as well as markers of inflammation (e.g. IL-6) and endothelial perturbation (e.g. vWf), could be an indicator of the severity of the ischemic episode in patients with acute coronary syndromes[195].

In numerous cardiovascular conditions, including the presence of common cardiovascular risk factors, the endothelium undergoes functional and structural modifications, compromising the functional integrity of the endothelium and ultimately leading to loss of its protective role[196].

Over the years, substantial experimental and clinical studies have linked endothelial dysfunction to human diabetes mellitus[197]. When talking about T2DM, the key mechanisms underlying the development of endothelial dysfunction include ROS production, inflammation, and chronic alterations of the hemodynamic balance[155].

Regarding the impact of diabetes mellitus on EPCs, CECs, and HSCs in patients with and without HFrEF, our sample size only allowed comparisons between patients with HFrEF with diabetes *versus* patients with HFrEF without diabetes. The small number of subjects with diabetes mellitus (n = 6) in the age-matched group precluded subgroup comparisons between groups and within this group.

Throughout the years, several authors have correlated endothelial damage with levels of circulating EPCs in patients with diabetes mellitus. Ultimately, concluding that EPCs found in patients with diabetes display several functional impairments, such as reduced proliferation, adhesion, and incorporation into tubular structures[198]. Worachat *et al.* reported

that the number of circulating EPCs was significantly decreased in patients with T2DM when compared to healthy subjects. Furthermore, they also observed that patients with adequate glycemic control had significantly higher levels of EPCs when compared to those with poor glycemic control[199]. Similarly, a study done by Fadini *et al.* assessed the mobilization of EPCs after ischemia-reperfusion injury in rats with streptozotocin-induced diabetes. They showed that bone marrow mobilization of EPCs, after ischemia-reperfusion injury, was defective in rats with diabetes. The defective mobilization of EPCs was associated with the downregulation of HIF-1α and the altered release of SDF-1 and VEGF, ultimately leading to insufficient compensatory angiogenesis[198,200]. Contrary to what was expected, we observed that patients from the HFrEF group with T2DM presented levels of EPCs similar to those of patients from the same group without T2DM. A possible explanation for this result may be due to the use of antidiabetic medications and good glycemic control, which have shown beneficial effects on EPC numbers and function[201]. Liang *et al.* cultured EPCs isolated from healthy individuals with various concentrations of advanced glycation end products with and without rosiglitazone treatment. They observed that rosiglitazone was able to reduce EPCs apoptosis, increase cell number, and enhance proliferation/migration capacity via upregulation of the Akt-eNOS signal pathways of EPCs[202].

Regarding CECs levels, our results showed that, when comparing subjects from the HFrEF group, CECs tended to circulate in a higher number in patients without T2DM compared to patients with T2DM[203]. However, CECs have been reported as emerging indicators of vascular injury and increased in T2DM. Indeed, a study performed by McClung *et al.* observed that levels of CECs were elevated in patients with T2DM compared to healthy controls. They concluded that the higher levels of CECs in patients with T2DM may reflect the existing vascular injury and is not directly dependent of the glucose control[204].

Lastly, when comparing HSCs levels between patients from the HFrEF group, similar levels were found between patients with and without T2DM. However, previous studies have shown a strong connection between mouse/mice models with diabetes mellitus and poor HSCs mobilization[205]. Oikawa *et al.* investigated the role of diabetes mellitus in microvascular remodeling and the consequences for bone marrow homeostasis. Subsequent detailed analysis of the bone marrow in streptozotocin-induced mice revealed microvascular rarefaction leading to decreased perfusion, endothelial barrier dysfunction, and reduced hematopoietic fraction with a decline in HSCs levels[206]. Another study performed by Spinetti

et al. showed that patients with T2DM, when compared with healthy controls, had lower levels of hematopoietic tissue, fat deposition, microvascular rarefaction, as well as a shortage of progenitor cells due to activation of proapoptotic pathways[207]. The different methodologies used to assess HSCs as well as the different models (animal *versus* human) may at least partially help to explain the discrepant results.

Endothelial dysfunction is an early, key aspect in the development of vascular disease, and is pathophysiologically linked to posterior atherosclerosis progression and cardiovascular diseases[208]. In cases of obesity, the overexpression of pro-inflammatory cytokines followed by a decrease of anti-inflammatory markers is considered to be the link between obesity-induced inflammation and endothelial dysfunction[209]. Over the past two decades, impaired endothelial function has been established in the vast majority of studies focusing on vascular damage in patients with obesity[210]. Regarding the impact of overweight/obesity on EPCs, CECs, and HSCs in patients with and without HFrEF, our sample size only allowed comparisons between patients with HFrEF diagnosed as overweight/obese *versus* patients with HFrEF with a normal weight. The small number of subjects with normal weight (n = 12) in the age-matched group precluded subgroup comparisons within this group. Our results showed that patients with HFrEF and diagnosed as overweight/obesity had higher levels of circulating EPCs and CECs compared to patients with HFrEF presenting a normal weight. Over the years contradictory results regarding the level and functionality of EPCs in patients with obesity have been reported[211]. In metabolically healthy obese patients with HF, the number of circulating EPCs is commonly increased or close to normal. On the other hand, metabolically unhealthy obese patients presented circulating EPCs with impaired proliferation, differentiation, migration, and adhesion capacities[212]. Tsai *et al.* found that the increase in the circulating number of EPCs, in response to lipopolysaccharide-induced endothelial damage, was remarkably suppressed in C57BL/6J mice given a high-fat diet[213]. This finding indicates that obesity diminished the circulating levels as well as the function of EPCs, impaired the recovery of damaged endothelium, suppressed EPC angiogenesis ability, and increased LV remodeling[214]. A study conducted in humans by Peterson *et al.* determined that female subjects with morbid obesity had a higher inflammatory state, as indicated by increased levels of TNFα, IL-6, leptin, Ox-HDL, EPCs, and CECs levels when compared to a group of subjects with no obesity[215]. The results from Peterson *et al*. are similar to ours, as we observed higher EPCs and CECs levels

in patients with HF diagnosed as overweight/obesity in comparison to patients with HF presenting a normal weight.

The role of the vascular endothelium in the development of hypertension is not easy to specify[216]. Activation of an alternative pathway involving cyclooxygenase, which leads to a decline of NO availability through the production of oxidative stress, is thought to be the mechanism responsible for the impairment of endothelial function in patients with hypertension[217]. Regarding participants with hypertension, our results have shown that EPCs levels were significantly lower in subjects with hypertension when compared with subjects without hypertension in the age-matched group. Furthermore, circulating EPCs levels were also significantly higher in subjects with hypertension, from the age-matched group, than in patients with hypertension from the HFrEF group. Over the years, many authors have focused on the relationship between blood pressure and EPCs levels, but with conflicting results[216]. For instance, Marketou *et al.* did not found any significant difference in the number of circulating EPCs between patients with hypertension and healthy subjects. However, pulse wave velocity, which is a reliable indicator of the stiffness of the large arteries, was found to be strongly correlated with circulating EPCs in patients with hypertension. This positive correlation suggests that EPCs could be mobilized into the circulation in response to vascular damage, preserving endothelial integrity[218]. Oliveras *et al.* reported that circulating EPCs levels were reduced by 56% in patients with refractory hypertension compared to healthy subjects[219]. Similarly, a study performed by Imanishi *et al.* found that, in patients with hypertension, the degree of hypertension-induced organ damage was positively correlated with EPCs senescence and negatively correlated with telomerase activity, which may affect the process of vascular remodeling[220].

When discussing CECs and HSCs levels in cases of hypertension, our study did not show any significant differences between patients with HFrEF and subjects presenting similar cardiovascular risk factors. However, increased levels of CECs as well as HSCs are referred to in the literature. For example, Budzyń *et al.* observed that patients with mild and resistant hypertension, as well as without left ventricular hypertrophy, had higher levels of CECs compared to healthy subjects[221]. Karthikeyan *et al.* found that pregnant women with hypertension presented higher levels of CECs compared to pregnant women without hypertension and non-pregnant healthy controls[222]. Seungbum *et al.* observed that mice with angiotensin-II induced hypertension presented higher proliferation of HSCs as evidenced by

an increase in Sca-1$^+$, c-Kit$^+$, and Lin$^-$ (SKL cells)[223]. Hashimoto *et al.* found that HSCs were increased in the peripheral circulation of patients with pulmonary arterial hypertension compared with normal individuals. In the same study, HSCs were increased in the bone marrow and the peripheral blood of mice with hypoxia-induced pulmonary hypertension in a time-dependent manner that coincides with a sudden rise in the large artery and ventricle stiffness[224].

Excessive lipids in the bloodstream can lead to endothelial dysfunction and are commonly described as a major risk factor for the development of cardiovascular diseases[225,226]. Since the majority of patients in the HFrEF group presented dyslipidemia (n = 34), the comparisons between subjects with and without dyslipidemia were only possible in the age-matched group. Therefore, our results showed that subjects with dyslipidemia had significantly higher levels of CECs compared to subjects without dyslipidemia. In addition, circulating EPCs and CECs levels were also significantly higher in subjects with dyslipidemia than in patients with dyslipidemia from the HFrEF group. However, contradictory results have been previously described. Pirro *et al.* observed that patients with hypercholesterolemia had lower levels of EPCs compared to patients without hypercholesterolemia. Additionally, Cheng *et al.* reported that EPC proliferative, migratory, and adhesive capacities were impaired in patients with high cholesterol levels [227–229].

Concerning CECs levels, a study done by Fabbri-Arrigoni *et al.* found that children's with familial hypercholesterolemia had higher levels of CECs when compared with healthy children's; with CECs levels similar to those found in healthy adults, implying that exposure to this vascular risk factor has an adverse impact on endothelial homeostasis, even at an early stage of life[230].

The present study has a number of limitations. First, this was a cross-sectional study in which we compared circulating EPCs, CECs, and HSCs levels between patients with HFrEF and age-matched apparently healthy subjects. However, we could not confirm whether the variation of EPCs, CECs, and HSCs number was the cause or the result of HFrEF and cardiovascular risk factors stated above. Second, the sample size was relatively small, which precludes us from drawing robust conclusions regarding the sub-comparisons between groups and within groups. A larger number of participants would allow us to explore other relationships, such as the possible association between obesity and levels of circulating EPCs. Third, 75% of the participants were men, future studies should include an

equal number of participants from both genders. Lastly, we were not able to assess EPCs, CECs, and HSCs functional capacities, such as adhesion, proliferation, and migratory ability.

CHAPTER VI: CONCLUSION AND FUTURE PERSPECTIVES

The main conclusion of this study is that the levels of circulating EPCs and CECs were significantly decreased in patients with HFrEF in comparison to subjects with cardiovascular risk factors. We did not confirm our hypothesis that increased numbers of CECs and decreased numbers of EPCs would be observed in patients with HFrEF per comparison to a group of subjects presenting similar cardiovascular risk factors but without established cardiovascular disease. Nevertheless, the higher CECs number observed in subjects with cardiovascular risk factors could suggest that patients with HFrEF may present highly controlled medical treatment compared to subjects with cardiovascular risk factors. However, these observations obtained from a cross-sectional study should be confirmed in a cohort study.

Future studies may explore novel methodologies for *in vitro* and *in vivo* evaluation of the functional characteristics of circulating EPCs, including their viability, adhesion, tube formation, and migration capacities[231]. Furthermore, recent advances in nanotechnology and tissue engineering for target delivery of cells, growth factors, or DNA may offer a great opportunity for successful EPCs delivery for therapeutic angiogenesis and tissue repair[232]. Upcoming studies should also evaluate the potential use of CECs for diagnosis and therapy monitoring by determining CECs levels during and after treatment in a wider range of cardiovascular diseases. However, several factors must be taken into consideration prior to integration into daily clinical practice, such as cost-effectiveness and appropriate serum level cut off[233]. Lastly, the enhancement of EPCs is considered a promising therapeutic approach for cardiovascular diseases. In recent years, several preliminary clinical trials have tested the safety and feasibility of transplantation of *ex vivo* EPCs for the treatment of patients with acute ischemic stroke[234]. Genetic modification of EPCs for targeted delivery of specific therapeutic agents or genes before transplantation may become a new research hot topic[235].

CHAPTER VII: REFERENCES

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